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(54) Title: APOPTOSIS

(57) Abstract: The present invention relates to the modulation of apoptosis, in particular it relates to the use of mTOR in the modulation of syncitial apoptosis. A method for the identification of further molecules involved in apoptosis, and the use mTOR in the treatment of disease are also disclosed. In addition, assays associated with the identification of such molecules are disclosed.

APOPTOSIS

FIELD OF THE INVENTION

The present invention relates to the modulation of apoptosis, in particular it relates to the use of mTOR in the modulation of syncitial apoptosis. A method for the identification of further molecules involved in apoptosis, and the use mTOR in the treatment of disease are also disclosed. In addition, assays associated with the identification of such molecules are disclosed.

BACKGROUND OF THE INVENTION

HIV-1 infection can cause apoptosis via a variety of mechanisms, some of which rely on the intricate virus host cell interaction (*in vitro*) and some of which involve activation of the host's inflammatory and immune systems (*in vivo*) (1.2). The envelope (Env) glycoprotein complex (gp120/gp41) appears to be one of the dominant apoptosis inducing molecules encoded by the HIV-1 genome. Env expressed on the plasma membrane of infected cells can interact with CD4 and a suitable co-receptor (*e.g.*, CXCR4) to trigger cell-to-cell fusion: the resulting syncytia subsequently undergo apoptosis. This applies to primary $CD4^+$ T lymphocytes inoculated by HIV-1 (3, 4), as well as to cell lines stably transfected with human CD4 co-cultured with cells expressing a lymphotropic HIV-1 *Env* gene (5-7). A positive correlation between $CD4^+$ T cell decline and infection by syncytium-inducing HIV-1 or SIV-1 variants has been established *in vitro* (3, 4, 8, 9) and, importantly, *in vivo*, in humans with AIDS (10, 11), humanized SCID mice (12) and monkeys (13). This suggests that fusion-induced apoptosis is relevant to AIDS pathogenesis, although syncytia are relatively infrequent in lymphoid tissues of HIV-1^+ donors (14). In lymph nodes from HIV-1 infected individuals, syncytia express markers of early apoptosis such as tissue transglutaminase (tTGase) (15). The scarcity of syncytia *in vivo* may be attributed to their rapid apoptotic degeneration and phagocytic removal.

Apoptosis-associated activation of caspases can be triggered by two pathways (16, 17). In the "extrinsic" pathway, the litigation of plasma membrane death receptors (e.g. CD95.TNF-R) leads to the recruitment of caspase-8 to the receptor complex, culminating in its proteolytic auto-activation (16). In the "intrinsic" pathway,

mitochondrial membrane permeabilization (MMP) results in the release of cytochrome *c* (Cyt.*c*) from the mitochondrial intermembrane space. Once in the cytosol, Cyt. *C* triggers the oligomerization of Apaf-1, which in turn recruits pro-caspase-9 and pro-caspase-3 into the apoptosome, the caspase activation multiprotein complex (18). MMP may be elicited by a variety of different pro-apoptotic second messengers (e.g. Ca^{2+}, ganglioside GD3, reactive oxygen species), as well as pro-apoptotic members of the Bcl-2 family such as Bax, Bak, Bid, and Bim (17).

Syncitial apoptosis induced by the interaction between Env and CD4/CXCR4, in the absence of viral infection, involves a mitochondrial pathway characterised by the following sequence of events: (i) translocation of Bax from the cytosol to mitochondria. (ii) Bax-mediated MMP with loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$), release of apoptogenic intermembrane proteins, in particular apoptosis inducing factor (AIF) and Cyt. *C* (iii) caspase activation, and (iv) nuclear chromatin condensation (6, 19).

Prior to the present invention, targets for modulating apoptosis, particularly syncitial apoptosis, were unknown. Methods for treating diseases associated with syncitial apoptosis, such as AIDS are needed.

Moreover, prior to the present invention, there was a need for diagnostic assays for prediction of predisposition to apoptosis, assays for identifying compounds capable of modulating p53S15P induction of apoptosis, and methods for identifying compounds capable of modulating mTOR apoptosis activity.

SUMMARY OF THE INVENTION

The present invention includes and provides inhibitor screening assays to identify and define molecules acting upstream of Bax, which result in the induction and translocation of Bax into the mitochondria leading to apoptosis, particularly syncitial apoptosis.

The present invention includes and provides a method for the modulation of apoptosis comprising the step of increasing, decreasing or otherwise altering the functional activity of mTOR/FRAP or a binding agent thereof.

By "modulating apoptosis" is meant that for a given cell, under certain environmental conditions, its normal tendency to undergo apoptosis is changed compared to an untreated cell such that apoptosis is increased or decreased relative to the normal rate. A decreased tendency to apoptose may also be a measurable increase in cell survival and may be the result of an inhibition of apoptosis by inhibiting one or more components of the apoptotic pathway. Conversely, an increased tendency or induction of apoptosis may be a measurable increase in cell death by apoptosis. Suitably, modulating apoptosis includes the induction of apoptosis.

Accordingly, in preferred embodiment of this aspect of the invention, there is provided a method for inducing apoptosis comprising the step of increasing, decreasing or otherwise altering the functional activity of mTOR/FRAP or a binding agent thereof.

The present invention also provides molecules including mTOR variants and fragments thereof as herein described.

The present invention further provides the use of mTOR, or an agent that alters the functional activity of mTOR in the modulation of apoptosis.

The present invention also provides a method for identifying a molecule involved in mediating apoptosis, comprising the steps, of: (a) providing one or more cells, (b) examining the one or more cells for an altered functional activity of mTOR, and/or p53, and/or the nucleic acid encoding them, and (c) assaying the one or more cells, and detecting those one or more molecule/s which interact with mTOR, and/or the nucleic acid encoding it.

The present invention also provides a method for identifying a molecule involved in mediating syncitial apoptosis, comprising the steps, of: (a) providing a cell population, wherein at least a proportion of the population have one or more syncitia present, (b) examining the one or more syncitia for an altered functional activity of mTOR, and/or p53, and/or the nucleic acid encoding them, and (c) assaying the one or more syncitia, and detecting those one or more molecule/s which interact with mTOR, and/or p53, and/or the nucleic acid encoding it.

The present invention includes and provides the use of mTOR, a binding agent thereof, or mTOR and p53, or a composition according to the present invention, in the treatment of a condition involving syncitial apoptosis.

The present invention provides and includes the use of one or more selected from the group consisting of: mTOR, a binding agent thereof, and mTOR and p53 in an assay for identifying an agent which modulates apoptosis and/or the cell size-related induction of apoptosis.

The present invention provides a diagnostic assay for predicting predisposition to apoptosis comprising: (i) detecting a level of a molecule selected from the group consisting of an mTOR molecule and a p53 molecule, wherein the p53 molecule is phosphorylated at serine 15; and (ii) comparing the level to a base-line amount.

The present invention also provides a method for identifying a compound capable of modulating p53S15P induction of apoptosis comprising: (i) obtaining a sample tissue or cell which expresses a p53 molecule; (ii) exposing the sample to the compound; and (iii) determining the level of a molecule selected from the group consisting of mTOR and p53, wherein the p53 molecule is phosphorylated at serine 15.

The present invention further provides a method for identifying a compound capable of modulating mTOR apoptosis activity comprising: (i) obtaining a sample tissue or cell which expresses a p53 molecule; (ii) exposing the sample to said compound; and (iii) determining the level of a molecule selected from the group consisting of mTOR and p53, wherein the p53 molecule is phosphorylated at serine 15.

The present invention also provides a method for identifying a compound capable of modulating mTOR apoptosis activity: (i) detecting a level of a molecule selected from the group consisting of an mTOR molecule and a p53 molecule, wherein the p53 molecule is phosphorylated at serine 15; and (ii) comparing the level to a base-line amount.

The present invention also provides a method for detecting apoptosis in a syncitia comprising detecting an increase in gene expression of transcription elongation factor B (SIII) or forkhead box E1.

The present invention also provides a method for detecting apoptosis in a syncitia comprising detecting a decrease in gene expression of E1B-55kDa-associated protein 5.

The present invention also provides a method for modulating apoptosis in a syncitia comprising the step of increasing, decreasing or otherwise altering the functional

activity of any one of transcription elongation factor B (SIII), forkhead box E1 or E1B-55kDa-associated protein 5.

DESCRIPTION OF THE FIGURES

Fig. 1: Expression levels of apoptosis-regulatory proteins and p53S15 phosphorylation in syncytia. **A.** Alteration in protein expression levels is determined by quantitative immunoblot analysis. The percent changes of the expression levels in Bax, Bid, and caspase-7 are shown after 18 and 36 hours of co-culture of HeLa Env and HeLa CD4 cells. Results are means of three independent determinations ± SEM. Asterisks indicate significant ($p<0.01$) changes. The right panel lists apoptosis-regulatory proteins which are detectable in HeLa cells and whose expression level did not change significantly (by less than 20%) within 36 hours after syncytium formation. **B.** Immunoblots of p53, p53Ser15P, Bax, and Bcl-2 at different intervals of syncytium formation. **C.** Immunofluorescence detection of p53S15P (red fluorescence, left panel) in syncytia counterstained with the nuclear dye Hoechst 33342 (blue fluorescence, right panel). **D.** Frequency of $p53S15P^+$ and apoptotic cells among syncytia (Syn) and single cells (SC) contained in co-cultures of HeLa Env and HeLa CD4 cells. **E.** Absence or presence of the conformational change of Bax detectable with a monoclonal antibody specific for the N-terminal Bax domain (green fluorescence) among 24-hour-old $p53S15P^+$ syncytia (red nuclear fluorescence). Certain cells lack Bax immunoreactivity (left) whereas others exhibit a cytoplasmic, in part punctuate staining pattern (right). **F.** Subcellular localisation of Cyt. *c* (green) in 24-hour-old $p53S15P^+$ syncytia (red). Mitochondrial (punctate) staining pattern (left) or the diffuse pattern (right) indicative of mitochondrial Cyt. *c* release. **G.** Quantitation of Bax and Cyt. *c* translocation among $p53S15P^-$ and $p53S15P^+$ syncytia, 24 or 48 hours after initiation of coculture. **H.** Effect of the pan-caspase inhibitor Z-VAD.fmk on p53S15 phosphorylation. Hela Env and HeLa CD4 cells are co-cultured for 24 hours in the presence or absence of Z-VAD.fmk, followed by determination of the frequency of $p53S15P^+$ cells and of cells exhibiting chromatin condensation. **I.** Effect of Bcl-2 on p53S15 phosphorylation. Syncytia (6h) are microinjected with recombinant Bcl-2 protein or sham-injected, and the frequency of

p53S15P$^+$ cells and nuclear apoptosis was determined at 24h. Values in H and I are means of triplicates ± SEM. The asterisks denote a significant ($p<0.01$) inhibition of chromatin condensation.

Fig. 2: Activation of p53 transcriptional activity in syncytia. **A.** Induction of MDM2 protein expression. Syncytia (18 h) are stained for p53S15P and MDM2. Representative cells are set forth. Strongly MDM2-positive (green) nuclear structures are only detected in p53S15P$^+$ (red) syncytia. Only a fraction of p53S15P$^+$ heterokarya are MDM2$^+$, **B.** Induction of p21 protein expression, as detected by immunofluorescence. p21$^+$ cells are a subpopulation of p53S15P$^+$ syncytia. **C.** Kinetics of positive immunostaining in syncytia for p53S15P, MDM2, p21, and Bax. Syncytia are subjected to immunofluorescence staining as in B, C. Results are mean values ±SD of three independent cultures. **D.** Expression of a p53-inducible reporter gene in HeLa cells. HeLa Env cells are transfected with the p53-inducible GFP construct alone or together with p53 or a p53 dominant negative mutant (p53H175) and cultured alone for 48 hours. Representative cells stained with Hoechst 33342 are shown. The percentage of control or p53H175-transfected cells exhibiting positive GFP fluorescence is <1%, that of p53-transfected cells 38±5% (n=3). **E.** Induction of p53-inducible GFP in syncytia. HeLa Env cells are transfected with a p53-inducible GFP construct (transfection efficiency ~40%) and fused 24 hours later with HeLa CD4 cells. A subpopulation of 24-hour-old p53S15P$^+$ syncytia (70 %) exhibited a diffuse GFP fluorescence.

Fig. 3: p53S15P in HIV-1 infection. **A.** p53S15P in HIV-1 infected syncytia. CD4-expressing HeLa cells are co-cultured with chronically HIV-1-infected H9/IIIB cells at a 3:1 ratio and stained for p53Ser15 (red), as well as the mitochondrial matrix protein hsp60 (green). The dominant phenotypes obtained 12 and 48 h after co-culture are shown. **B.** p53S15P in uninfected or HIV-1 infected U937 cells, as determined by immunoperoxidase staining (brown) and hematoxylin counterstaining (blue) and confirmed by immunoblot. **C.** p53S15P$^+$ syncytium in the apical light zone of lymph node derived from an HIV1$^+$ donor (arrow). On a consecutive section tTgase expression

is detected. The insert show a p53S15P$^+$ syncytium with condensed nuclei. Similar pictures are obtained for 5 different HIV-1$^+$donors. No p53S15P$^+$ cells are detected in HIV-1$^-$ controls (lower panel). **D.** 53S15P in PBMC is derived from representative HIV-1$^-$ or HIV-1$^+$ donors. Immunoperoxidase staining and immunoblots are shown. **E.** Correlation between p53S15P and tTgase expression in PBMC from 49 HIV-1$^+$ patients. The frequency of p53S15P$^+$ and tTgase$^+$ cells is determined by immunocytochemical analysis of PBMC from 49 HIV-infected individuals, at least 2 months after interruption of antiretroviral therapy (as in D). Each dot represents one HIV-infected patient. Statistics are calculated by means of the Pearson method for parametric data using the Graph Pad Prism statistical program. The correlation coefficient (Pearson r) is 0.7107. **F.** Correlation between p53S15P and viral load of the same patients as in E. The correlation coefficient is 0.3922.

Fig. 4: Mechanism of p53S15 phosphorylation. **A.** Alterations in the expression level of serine kinases and phosphatases are determined by quantitative immunoblot analysis. The percent changes of the expression levels in mTOR/FRAP and PP2a are shown (X±SEM, n = 3). Asterisks indicate significant ($p<0.01$) changes. The right panel lists serine kinases and phosphatases, as well as related proteins, which are detectable in HeLa cells and whose expression level varied less than 20% within 18 or 36 hours after syncytium formation. **B.** Inhibitory profile of p53S15 phosphorylation. HeLa Env and HeLa CD4 cells are cocultured for 24 hours in the presence of the indicated agents, followed by determination of the frequency of p53S15P$^+$ syncytia. Results are means of 3 to 5 independent determinations ± SEM. Asterisks indicate significant ($p<0.01$, Student t test) inhibitory effects. **C.** Representative comet assay results obtained for untreated HeLa CD4$^+$ single cells (SC, negative control), H_2O_2-treated HeLa CD4$^+$ cells (positive control), adherent Env/CD4-induced syncytia (18 hours) or non-adherent, apoptotic Env/CD4-induced syncytia. **D.** Quantitation of comets (which reveal double strand breaks), as determined by image analysis (X±SD, n = 100). **E.** Effect of micro-injected mAb specific for mTOR/FRAP on p53S15P. Six hours after fusion, syncytia were microinjected with mAbs specific for mTOR/FRAP or LAMP-1 (negative control),

cultured for further 24 hours and subjected to p53S15P staining. The asterisk indicates a significant ($p<0.01$) inhibitory effect. Results are mean values ± SEM of three experiments.

Fig. 5: Biological effects of FRAP on syncytia. **A.** Phosphorylation of the $p70^{s6k}$ protein kinase, 4E binding protein 1 (4EBP1), and p53S15 in syncytia. A 1:1 mixture of HeLa Env and HeLa CD4 single cells (lane 1), 24-hour-old syncytia cultured in the absence (lane 2) or presence (lane 3) of 1 μM rapamycin, 30 h-serum-deprived Env cells before (lane 4) or after addition of serum for 3 hours (lanes 5 and 6), in the presence (lane 5) or absence of 1 μM rapamycin (lane 6) are subjected to SDS-PAGE followed by immunodetection of phosphorylated $p70^{s6k}$ (using a phospho-specific mAb), 4E binding protein 1 (phosphorylated protein exhibits reduced electrophoretic mobility), or p53S15P. **B**. TOP-dependent translation in syncytia. HeLa Env cells are transiently transfected with a luciferase construct containing TOP in the promoter region or a control construct lacking TOP. These cells are mixed with an equivalent amount of HeLa CD4 cells without co-culture (1), or co-cultured with HeLa CD4 cells for 24 hours, in the absence (2) or presence (3) of 1 μM rapamycin. Alternatively, the cells are serum-deprived for 30 hours and then cultured for additional 3 hours in the absence (4) or in the presence (5,6) of serum, with (6) or without (5) 1 μM rapamycin. Cellular extracts (50 μg per point) are prepared and luciferase activities determined. TOP-dependent luciferase activity is divided by the TOP-independent luciferase activity and normalized assuming that single cells cultured in the presence of serum and in the absence of rapamycin (1, 5) represent 100% TOP-dependent translation. **C**. Co-immunoprecipitation of mTOR/FRAP and $p53S15P^{+}$ in syncytia. Ad hoc mixtures of HeLa Env and HeLa CD4 single cells (SC), 24 h-old syncytia (Syn) are lysed and then subjected to immunopreciptation with antisera specific for p53, p53S15P or Bcl-2 (negative control), SDS-PAGE, and immunodetection of mTOR/FRAP. **D.** Enhanced expression of mTOR/FRAP in syncytia. As compared to single cells (arrows), syncytia manifest an enhanced, mainly nuclear staining for mTOR/FRAP. Two 12-hour-old syncytia representing major phenotypes are shown: $mTOR/FRAP^{high}p53Ser15P^{-}$ and $mTOR/FRAP^{high}p53Ser15P^{+}$. Note that all

p53Ser15P$^+$ syncytia are mTOR/FRAP^{high}, mTOR/FRAP^{high} syncytia are either p53Ser15P$^+$ or p53Ser15P$^-$. **E.** Quantitation of the frequency of mTOR/FRAP^{high} and p53Ser15P$^+$ cells at different time points.

Fig. 6: Apoptosis inhibition by rapamycin and LY294002 in Env/CD4-induced syncytia. **A.** Representative JC-1/Hoechst 33342 staining of syncytia generated by 48 h of coculture in the absence (control) or presence of 1 μM rapamycin. Cells are stained with Hoechst 33342 (blue fluorescence) and the $\Delta\Psi_m$-sensitive dye JC-1 (red fluorescence of mitochondria with a high $\Delta\Psi_m$, green fluorescence of mitochondria with a low $\Delta\Psi_m$). Rapamycin antagonizes the $\Delta\Psi_m$ loss, as well as chromatin condensation. **B.** Inhibition of mitochondrial and nuclear signs of apoptosis. Cocultures (48 h) of HeLa Env and HeLa CD4 cells are performed in the presence of rapamycin or LY294002, followed by quantitation of the $\Delta\Psi_m$ loss and chromatin condensation on live cells (determined as in A) or, alternatively, permeabilization, fixation and immunostaining with an anti-Bax or anti-Cyt. *c* antibody (as in Fig. 1 e,f). Asterisks indicate significant ($p<0.01$) inhibitory effects. **C.** Quantitation of the number of nuclei per syncytium (n=100) after 72 hours of coculture. Pan-caspase inhibitor Z-VAD.fmk (100 μM, added at the beginning of coculture) is included as a positive control. Asterisks indicate significant ($p<0.01$) effects. **D.** Effects of apoptosis-regulatory compounds on syncytia. The effect of different agents on nuclear apoptosis are assessed as in A. "No effect" denotes less than 20% inhibition or enhancement of chromatin condensation. "Toxicity" and "protection" denote >20% increase or reduction of apoptosis, respectively. PDTC inhibited apoptosis by 52±6 % in three independent experiments.

Fig. 7: Apoptosis inhibition by dominant negative p53. Both HeLa Env and HeLa CD4 cells are transfected by p53H175 or p53 H273, as well as a vector control only (Co.). 24 hours after the transfection the cells are co-cultured for 48 hours and the mitochondrial and nuclear parameters indicative of apoptosis are assessed by JC1/Hoechst 33342 or immunofluorescence staining. Asterisks indicate significant ($p<0.01$) inhibitory effects, as compared to vector-only-transfected cells. The insert demonstrates the transfection-

enforced overexpression of mutant p53, as determined by immunoblotting. Results are representative of four independent determinations.

Fig. 8: p53 is associated with syncytial apoptosis. Wild type ($p53^{+/+}$), $p53^{+/-}$ and $p53^{-/-}$ cells are fused by transient exposure to PEG. **A.** Syncytia (9 h) arising from $p53^{+/+}$ MEF showing positive immunoreactivity after staining for p53S15P or Bax. Bax^{+} cells are also positive for p53S15P. As a control, a representative $p53^{-/-}$ syncytium (which stays Bax^{-}) is shown. **B.** Quantitation of apoptosis. Syncytia arising from the fusion of $p53^{+/+}$, $p53^{+/-}$ or $p53^{-/-}$ cells are stained at the indicated time points with JC1/Hoechst 33342 (as in Fig. 6a) and the frequency of cells exhibiting a low $\Delta\Psi_m$ and chromatin condensation is determined. Results are representative of three independent experiments.

Fig. 9: Rapamycin effects on syncytial apoptosis induced by HIV-1 infection. **A.** Fluorescence micrographs of HeLa CD4 cells cocultured with HIV-1-infected H9/IIIB cells for 48 hours in the absence (Co.) or the presence of 1 μM rapamycin. Cells are stained with Hoechst 33342 and the $\Delta\Psi_m$-sensitive dye JC-1 (as in Fig. 6a). Primary $CD4^{+}$ lymphoblasts infected with HIV-1 IIIb are also shown. **B.** Rapamycin effects on p53S15P and apoptosis in HeLa CD4 cells cocultured with H9/IIIB cells. p53S15P is determined by immunofluorescence as in Fig 1c and apoptosis is measured by staining with JC-1/Hoechst. Values refer to the percentage of syncytia that are $p53S15P^{+}$ or apoptotic. Asterisks denote significant ($p<0.01$) effects of rapamycin, as compared to untreated cocultures. **C+D.** Rapamycin-enhanced viability of syncytia associated with apoptosis inhibition. CEM cells stably transfected with a Tat-inducible GFP or HIV-1-infected H9/IIIB cells are cultured alone or co-cultured (CC), in the absence or presence of AMD3100 (1 μg/ml), Z-VAD.fmk (50 μM), or rapamycin (1μM). GFP-dependent fluorescence (arbitrary units, a.u.) is measured to assess the total syncytial mass (C). Parallel cultures are subjected to lysis, staining with propidium iodine, and cytofluorometric determination of the percentage (X±SEM, n=3) of hypoploid (sub-G_1) nuclei (D). **E.** Rapamycin effects on apoptosis of HIV-1 infected primary $CD4^{+}$

lymphoblasts. Two days after infection with HIV-IIIB, the inhibitors AMD3100 (which prevents the Env/CD4 interaction, 5 μg/ml), Z-VAD.fmk (100 μM) or rapamycin are added to the cultures. At day 4, the production of p24 (<0.5 ng/ml on day 2) is determined. In addition, the frequency of syncytia with condensed nuclei and the number of nuclei per syncytium are determined. Results are mean values ± SEM of three independent experiments. Asterisks denote significant ($p<0.01$) effects as compared to control cultures.

Fig. 10a: shows that global changes in gene expression are associated with Env-induced syncytial apoptosis, by cluster analysis of LifeGrid filters. HeLa cells transfected with the Env gene of HIV-1 LAI (HeLa Env. (27)) and HeLa cells transfected with CD4 (HeLa CD4 (5)) are cultured alone (**C; CONTROL**) or together (1:1 ratio. **F; FUSION**) in medium supplemented with 10% FCS, essentially as described (in references 5, 6, and 27). Samples are isolated for RNA extraction and microarray gene analysis, 2 h, 4 h, 8 h and 16 h. Average fold change values (from two spots on the filters) for Controls and Fusions, are with respect to time = 2h values respectively, and are analysed by GeneMaths using a Pearson correlation and Ward cluster algorithms. Increased expression (light) and decreased expression (dark) are represented and referenced by a gray scale bar.

Fig 10b: shows a graphical representation of genes whose expression change is associated with Env-induced syncytial apoptosis. HeLa cells transfected with the Env gene of HIV-1 LAI (HeLa Env. (27)) and HeLa cells transfected with CD4 (HeLa CD4 (5)) are cultured alone (**C; CONTROL**) or together (1:1 ratio. **F; FUSION**) in medium supplemented with 10% FCS, essentially as described (in references 5, 6, and 27). Samples are isolated for RNA extraction and microarray gene analysis, 2 h, 4 h, 8 h and 16 h. Average fold change values (from two spots on the filters) for Controls and Fusions, are with respect to time = 2h values respectively. Two genes (transcription elongation factor B (SIII; **light**) and forkhead box E1 (**dark**) are illustrated; whose expression increases with Env-induced syncytial apoptosis (see F vs. C).

Fig 10c: shows a graphical representation of genes whose expression change is associated with Env-induced syncytial apoptosis. HeLa cells transfected with the Env gene of HIV-1 LAI (HeLa Env. (27)) and HeLa cells transfected with CD4 (HeLa CD4 (5)) are cultured alone (**C; CONTROL**) or together (1:1 ratio. **F; FUSION**) in medium supplemented with 10% FCS, essentially as described (in references 5, 6, and 27). Samples are isolated for RNA extraction and microarray gene analysis, 2 h, 4 h, 8 h and 16 h. Average fold change values (from two spots on the filters) for Controls and Fusions, are with respect to time = 2h values respectively. A genes (E1B-55kDa-associated protein 5) is illustrated; whose expression decreases with Env-induced syncytial apoptosis, relative to control (see F vs. C).

DEFINITIONS

As used herein, "apoptosis" or "cell death" may occur by accident, by cell necrosis, or by a controlled intracellular process characterised by the condensation and subsequent fragmentation of the cell nucleus during which the plasma membrane remains intact. A cascade of enzymes including caspases that cleave at aspartic acid residues become activated during the process.

As used herein, a "syncytium" is a mass of cytoplasm containing several nuclei enclosed within a single plasma membrane. Syncitia are normally derived from single cells that fuse or fail to complete cell division. Examples include muscle fibres, formed by the fusion of myoblasts, and residual bodies formed during spermatogenesis. As used and exemplified herein, the term "syncytium" or "syncitia" also includes any fusion between cells. Such a fusion of cells can be between cells of the same type or of different types. Suitably, a "syncitia" can be formed between two non tumour-derived cells. In one embodiment, a "syncitia" can be formed by the fusion of a tumour cell and a normal cell as found, for example, in a hybridoma where a tumour cell is fused to a normal cell, normally a lymphocyte, to create a cell line *in vitro*.

As used herein, "syncitial apoptosis" describes a form of apoptosis that involved the formation of syncitia prior to the apoptosis event. Syncitia formation can be induced

by the cross-reaction of certain receptors on cell surfaces, for example if two different Hela cell populations, one transfected with HIV-1 env surface protein, and the other with the CR4/CXR4 receptor are mixed syncitia and apoptosis will spontaneously result. Syncitial apoptosis can involve a signal transduction pathway involving the translocation of Bax from the cytosol to the miotchondria and the release of apotogenic intermembrane proteins, including apoptosis inducing factor and cyt C.

As used herein, "cell size-related induction of apoptosis" includes within its scope the induction of apoptosis due to a cell becoming overly large, or due to an altered volume/surface ratio of a cell as compared with a normal cell. External constraints on cell size and shape have a profound effect on HIV-1 induced syncitial apoptosis.

As used herein, "functional activity" of a protein in the context of the present invention describes the function the protein performs in its native environment. Altering the functional activity of a protein includes within its scope increasing, decreasing or otherwise altering the native activity of the protein itself. In addition, it also includes within its scope increasing or decreasing the level of expression and/or altering the intracellular distribution of the nucleic acid encoding the protein, and/or altering the intracellular distribution of the protein itself.

In the context of the present invention the term "altered functional activity of mTOR" includes within its scope increasing, decreasing or otherwise altering the native activity of the protein itself. In addition, it also includes within its scope increasing or decreasing the level of expression and/or altering the intracellular distribution of the nucleic acid encoding the protein, and/or altering the intracellular distribution of the protein itself.

In the context of the present invention the term "a condition involving syncitial apoptosis" means any condition whether pathological or non-pathological which has at least as a component syncitia formation and subsequent apoptosis.

As used herein, the term "presence" refers to when a molecule can be detected using a particular detection methodology. Also as used herein, the term "absence" refers to when a molecule cannot by detected using a particular detection methodology.

As used herein, references to the molecule mTOR and p53 includes within its scope, variants, derivatives and fragments thereof, as herein defined.

As used herein, "p53S15P" or "p53 serine 15P", "p53S15P+" refer to a p53 kinase where the fifteenth serine is phosphorylated. "p53S15P-" refers to p53 kinase where the fifteenth serine is not phosphorylated

DETAILED DESCRIPTION OF THE INVENTION

General Techniques

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel et al., Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc.) and chemical methods. In addition, Harlow & Lane, A Laboratory Manual Cold Spring Harbor, N.Y, is referred to for standard Immunological Techniques.

MTOR, p53S15P and binding agents thereof

mTOR

A model cell system described in Ferri *et al* (6) can be used as a basis to screen for molecules which act upstream of Bax, and result in the induction and mitochondrial translocation of Bax and subsequent syncitial apoptosis. Standard techniques such as proteomics and inhibitor screening assays including without limitations herein described, may be employed to identify those molecules that interact with, and alter the functional activity of Bax.

An mTOR protein (mammalian target of rapamycin) (mTOR is also referred to as FKBP12-rapamycin associated protein (FRAP)) has been identified using such an assay. mTOR exerts translational control via the phosphorylation of p70s6k protein kinase and the 4E binding protein I (23, 24, 52). mTOR/FRAP is further known to inhibit a serine phosphatase PP2A (48).

In a first aspect the present invention provides a method for the modulation of apoptosis comprising the step of increasing, decreasing or otherwise altering the

functional activity of mTOR/FRAP, or a binding agent thereof. In a preferred aspect, the apoptosis is synctial apoptois.

In the context of the present invention the term mTOR also includes within its scope, variants, derivatives and fragments thereof, in as far as they possess the requisite ability to modulate apoptosis.

The terms "variant" or "derivative" in relation to the amino acid sequences of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence. Preferably, nucleic acids according to the invention are understood to comprise variants or derivatives thereof.

Natural variants of mTOR are likely to comprise conservative amino acid substitutions. Conservative substitutions may be defined, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Suitable fragments of mTOR will be at least about 5, 10, 12, 15 or 20 amino acids in length. They may also be less than 100, 75 or 50 amino acids in length. They may contain one or more (preferably. 5, 10, 15, or 20) substitutions, deletions or insertions, including conserved substitutions. A fragment of mTOR according to the present invention must possess the requisite activity of being capable of being able to modulate apoptosis.

mTOR binding agents

Suitable molecules/agents may be naturally occurring or synthetic They include molecules which bind either directly or indirectly to the protein, or to the nucleic acid encoding it, in order to modulate the proteins functional activity as herein defined. Agents may be naturally occurring molecules such as peptides and proteins, for example antibodies, or they may be synthetic molecules. One skilled in the art will appreciate that this list is not intended to be exhaustive. In a preferred embodiment of the invention, the agent is Rapamycin or analogues thereof, more preferably Rapamycin. In a further preferred embodiment the agent is an antibody raised against mTOR/FRAP.

Antibodies suitable for use according to the present invention may be monoclonal or polyclonal. Methods for their preparation will be familiar to those skilled in the art. For example, without limitation, either recombinant proteins or those derived from natural sources can be used to generate antibodies. For example, the protein (or "immunogen") is administered to challenge a mammal such as a monkey, goat, rabbit or mouse. The resulting antibodies can be collected as polyclonal sera, or antibody-producing cells from the challenged animal can be immortalised (e.g. by fusion with an immortalising fusion partner to produce a hybridoma), which cells then produce monoclonal antibodies.

In an alternative embodiment, one or more agents which bind to mTOR may be added to the syncitia in a method of the present invention, and the change in the functional activity of mTOR detected. Suitable molecules include Rapamycin and p53, and their analogues. Suitable methods for detecting the resultant change in the functional activity of mTOR and/or a binding agent thereof, include but are not limited to those described herein.

a. Polyclonal antibodies

The antigen protein is either used alone or conjugated to a conventional carrier in order to increases its immunogenicity, and an antiserum to the peptide-carrier conjugate is raised in an animal, as described above. Coupling of a peptide to a carrier protein and immunizations may be performed as described (Dymecki *et al.* (1992) J. Biol. Chem., **267**: 4815). The serum is titered against protein antigen by ELISA or alternatively by dot

or spot blotting (Boersma and Van Leeuwen (1994) J. Neurosci. Methods, **51**: 317). The serum is shown to react strongly with the appropriate peptides by ELISA, for example, following the procedures of Green *et al.* (1982) Cell, **28**: 477.

b. Monoclonal antibodies

Techniques for preparing monoclonal antibodies are well known, and monoclonal antibodies may be prepared using any candidate antigen, preferably bound to a carrier, as described by Arnheiter *et al.* (1981) Nature, **294**, 278. Monoclonal antibodies are typically obtained from hybridoma tissue cultures or from ascites fluid obtained from animals into which the hybridoma tissue was introduced. Nevertheless, monoclonal antibodies may be described as being "raised against" or "induced by" a protein.

After being raised, monoclonal antibodies are tested for function and specificity by any of a number of means. Similar procedures can also be used to test recombinant antibodies produced by phage display or other in vitro selection technologies. Monoclonal antibody-producing hybridomas (or polyclonal sera) can be screened for antibody binding to the immunogen, as well. Particularly preferred immunological tests include enzyme-linked immunoassays (ELISA), immunoblotting and immunoprecipitation (see Voller, (1978) Diagnostic Horizons, **2**: 1, Microbiological Associates Quarterly Publication, Walkersville, MD; Voller et al. (1978) J. Clin. Pathol., **31**: 507; U.S. Reissue Pat. No. 31,006; UK Patent 2,019,408; Butler (1981) Methods Enzymol., **73**: 482; Maggio, E. (ed.), (1980) Enzyme Immunoassay, CRC Press, Boca Raton, FL) or radioimmunoassays (RIA) (Weintraub, B., Principles of radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March 1986, pp. 1-5, 46-49 and 68-78). Techniques for labelling antibody molecules are well known to those skilled in the art (see Harlow and Lane (1989) Antibodies, Cold Spring Harbor Laboratory, pp. 1-726.)

Detecting an altered functional activity of mTOR

Standard laboratory techniques such as immunoblotting can be used to detect altered levels of mTOR within syncitia, as compared with single cells in the same cell population. An example of a suitable protocol is detailed below:

Aliquots of total protein extracts (40μg), are run on SDS-PAGE and electroblotted overnight at 4°C onto nitrocellulose membrane. Immundetection involved antibodies specific for p53, Bax, Bcl-2, p53, m TOR/FRAP (BD Transduction laboratories), p53S15P (Cell Signalling Technology, MA, USA), appropriate secondary antibodies (goat, anti-rabbit or goat-anti-mouse: Bio-Rad, CA, USA) conjugated to horseradish peroxidase, and the enhanced ECL chemiluminescence detection system (Amersham, UK).

For immunoprecipitation, lysates from sonicated, Triton X-100-solublized cells (60μg protein in 100μl PBS with protease inhibitors) are incubated for 90 min at 37°C with 500 ng affinity-purified rabbit polyclonal antibodies specific for p53, p53S15P or Bcl-2, followed by an addition of 10μl packed protein A/G-agarose beads (30 min, 37°C: Santa Cruz Biotechnology), vigorous washing of the pellet (10 min at 10000g, 3 x) in PBS, 5% SDS PAGE, and immunodetection with an m TOR FRAP-specific mAb. Characteristics of mTOR such as immunoprecipitation and phosphorylation activity can be used as the basis of an assay to examine the syncitia for an altered functional activity of mTOR. An example of a protocol for immunoprecipitation is detailed herein:

Methods for detecting the phosphorylation of a protein are known to those skilled in the art and include the use of phosphate specific antibodies. For example the phosphorylation of p53 at serine 15 can be detected using a panel of phosphoserine specific antibodies, for example, without limitation as herein described.

Techniques such as analytical centfiugation, affinity binding studies involving chromatography or electrophoresis can be used to detect molecules which interact directly with mTOR. Those skilled in the art will appreciate that this list is by no means exhaustive. More specifically, it is possible to use mTOR as an affinity ligand to identify agents which bind to it; labeling mTOR with a detectable label and using it as a probe to detect apoptotic products, particularly syncitial apoptotic products on blots of electrophoresis gels; labeling the mTOR target and using it to probe libraries of genes and/or cDNAs; labeling the mTOR target and using it to probe cDNA expression libraries derived from syncitia or apoptotic cells to find clones synthesizing proteins which can bind to the target; performing UV-crosslinking studies to identify agents which can bind to the target; using the mTOR in gel retardation assays which would detect its ability to

bind to nucleic acid encoding identified agents; performing footprinting analyses to identify the regions within a nucleic acid to which the target binds. Those skilled in the art will be aware of other suitable techniques and will appreciate that this list is not intended to be exhaustive.

In addition, changes in events immediately down-stream of mTOR, such as the phoshorylation of serine 15 of p53, can be used as an indication that a molecule in question affects the functional activity of mTOR.

Molecular Techniques

Techniques such as phage display and yeast two-hybrid technology (X) can be employed in the identification of candidate binding agents which interact with mTOR.

(I) Phage display

Phage display is a protocol of molecular screening which utilises recombinant bacteriophage. The technology involves transforming bacteriophage with a gene that encodes an appropriate ligand (in this case mTOR or a derivative or variant thereof) capable of reacting to specific syncitial or cellular components. The transformed bacteriophage (which preferably is tethered to a solid support) expresses the appropriate ligand (such as mTOR) and displays it on their phage coat. The entity or entities (such as syncitial components) which recognises the candidate agent (in this case mTOR) are isolated and amplified. The successful components are then characterised. Phage display has advantages over standard affinity ligand screening technologies. The phage surface displays the candidate agent in a three dimensional configuration, more closely resembling its naturally occurring conformation. This allows for more specific and higher affinity binding for screening purposes.

(II) Yeast two-hybrid system

Two hybrid methods provide a simple and sensitive means to detect the interaction between two proteins in living cells. All systems share common elements. All use 1) a plasmid that directs the synthesis of a "bait": a known protein which is brought to DNA by being fused to a DNA binding domain, 2) one or more reporter genes

("reporters") with upstream binding sites for the bait, and 3) a plasmid that directs the synthesis of proteins fused to activation domains and other useful moieties ("activation tagged proteins", or "prey"). All current systems direct the synthesis of proteins that carry the activation domain at the amino terminus of the fusion, facilitating the expression of open reading frames encoded by cDNAs. Figure 1 illustrates many of these elements.

The systems differ in their specifics. These details are typically relevant to their successful use. Baits differ in their DNA binding domains. Some systems use baits that contain native E. coli LexA repressor protein. LexA binds tightly to appropriate operator, and carries a dimerization domain at its C terminus. In yeast, LexA and most LexA derivatives enter the nucleus, but are not necessarily nuclear localized. Others use baits that contain a portion of the yeast GAL4 protein. This portion, encoded by residues 1-147, is sufficient to bind tightly to appropriate DNA binding sites, localize fused proteins to the nucleus, and direct dimerization; it also contains a domain that weakly activates transcription from mammalian cell extracts *in vitro*.

Reporter genes differ in the phenotypes they confer. The products of some reporter genes (e.g., HIS3, LEU2) allow cells expressing them to be selected by growth on appropriate medium, while the products of others (e.g. lacZ) allow cells expressing them to be visually screened. Reporters also differ in the number and affinity of upstream binding sites (e.g., lexA operators) for the bait, and in the position of these sites relative to the transcription start point. Finally, they differ in the number of molecules of the reporter gene product necessary to score the phenotype. These differences affect the strength of the protein interactions the reporters can detect.

Preys differ in the activation domains they carry, and in whether they contain other useful moieties such as nuclear localization sequences and epitope tags. Some activation domains are stronger than others. Although strong activation domains should allow detection of weaker interactions, their expression can also harm the cell due to poorly understood transcriptional effects, either by titration of cofactors necessary for transcription of other genes ("squelching") or by toxic effects that result when strong activation domains are brought to DNA. Activation tagged proteins also differ in whether they are expressed constitutively, or conditionally. Conditional expression allows the

transcription phenotypes obtained in selections (or "hunts") for interactors to be ascribed to the synthesis of the tagged protein, thus reducing the number of false positive cells that grow because their reporters are aberrantly transcribed.

Uses of agents and molecules of the present invention

mTOR, binding agents thereof, mTOR and p53, and compositions according to the present invention can, for example, be employed in *in vivo* therapeutic and prophylactic applications, *in vitro* and *in vivo* diagnostic applications, *in vitro* assay and reagent applications.

Therapeutic and prophylactic uses of molecules and compositions according to the invention involve the administration of the above to a recipient mammal, such as a human.

Substantially pure molecules of at least 90 to 95% homogeneity are preferred for administration to a mammal, and 98 to 99% or more homogeneity is most preferred for pharmaceutical uses, especially when the mammal is a human. Once purified, partially or to homogeneity as desired, the molecules used diagnostically or therapeutically (including extracorporeally) or in developing and performing assay procedures using methods known to those skilled in the art.

Molecules of the present invention will find use in modulating conditions in which apoptosis is involved, particularly synctial apoptosis. These typically include conditions such as AIDS, cancer, age related bone loss, osteoporosis, leukemia, skeletal muscle disorders, coronavirus infection, hepatitis, measles and transplant rejection. For example without limitation the administration of Rapamycin, or one or more molecules which inhibit the functional activity of mTOR, to a patient will substantially inhibit apoptosis, particularly syncitial apoptosis in that patient, and may therefore be used in the treatment or the prevention, or the suppression of conditions such as AIDS. Alternatively, the administration of mTOR, a variant, derivative or fragment thereof to a patient will promote apoptosis, particularly syncitial apoptosis and may be used in the treatment, suppression, or prevention of conditions such as measles and hepatitis, certain skeletal muscle disorders and conditions relating to bone loss in mammals.

Syncitial apoptosis has been implicated in a number of pathological and non-pathological conditions. For example, murine coronavirus, MHV-3 is an example of an RNA-containing virus capable of inducing apoptosis. In this study apoptosis was inversely correlated with the development of typical MHV cytopathology, namely syncytia formation. In addition, the pattern of induction of apoptosis during infection with MHV-3 correlates with strain variation in resistance and susceptibility to lethal hepatitis (Belyavskyi, M et al, Adv. Exp.Med. Biol. 1998: 440: 619-25).

Measles virus suppresses cell-mediated immunity by interfering with the survival and functions of dendritic and T cells.. The interaction of measles virus-infected dentritic cells with T cells not only induces syncytia but also leads to an impairment of dentritic cell and T cell function and cell death. (Fugier-Vivier I et al, J. Exp Med 1997 Sept 15, 186(6): 813-23)

Apoptosis and syncytial fusion occur in human placental trophoblast and skeletal muscle. Skeletal muscle fibres and placental villous trophoblast are the main representatives of syncytia in the human. Both syncytia are derived from fusion of mononucleated stem cells, show a high degree of differentiation, and have lost their generative potency. As early as in the differentiated stages of the mononucleated stem cells, initiation stages of the apoptosis cascade have been observed. After syncytial fusion progression of the cascade is retarded or blocked by a variety of mechanisms (Huppertz B et al, Int Rev Cytol. 2001)

Syncitia have also been implicated in bone formation and remodelling. Osteoclasts are giant multinucleated cells responsible for bone resorption. The first events during bone remodeling is osteoclast activation, followed by osteoclast formation, polarization constitution of the ruffled border, resorption and ultimately apoptosis. Osteoclast apoptosis is followed by a series of sequential changes in cells in the osteoblast lineage, including osteoblast chemotaxis, proliferation and differentiation, which in turn is followed by formation of mineralized bone and cessation of osteoblast activity. A molecule of the present invention may thereof be of use in the modulation of syncitial apoptosis relating to bone loss which occurs in age and in several pathological conditions (Brandi ML, Masi L, QJ Nucl Med 2001, Mar 45 (1) 2-6).

Regulatory apoptotic proteins are expressed in peripheral giant cell granulomas and lesions containing osteoclast-like giant cells. The frequency of apoptotic nuclei detected by TUNEL-staining compared to regular nuclei was 18 times higher in giant cells than in mononuclear cells, suggesting that syncitial apoptosis may be occurring in these cells.

In the context of the present invention the term 'a condition involving syncitial apoptosis' means any condition whether pathological or non-pathological which has at least as a component syncitia formation and subsequent apoptosis.

In a preferred embodiment of this aspect of the invention, the condition is selected from the group consisting of: AIDS, cancer, age related bone loss, osteoporosis, leukemia, and skeletal muscle disorders.

Thus, in a further aspect, the present invention provides the use of mTOR, or a binding agent thereof, or mTOR and p53 in the treatment of conditions involving the cell size related induction of apoptosis.

The term 'cell size-related induction of apoptosis' includes within its scope the induction of apoptosis due to a cell becoming overly large, or due to an altered volume/surface ratio of a cell as compared with a normal cell. External constraints on cell size and shape have a profound effect on HIV-1 induced syncitial apoptosis. The present inventors believe that an abnormal size and/or distorted volume/surface ratio of cells, activates the mTOR/FRAP pathway and causes cellular demise.

Preferably the condition is one or more selected from the group consisting of: granulomas, giant cell tumours, giant cell hepatitis and giant cell arthritis. One skilled in the art will appreciate that this list is not intended to be exhaustive.

In the instant application, the term "prevention" involves administration of the protective composition prior to the induction of the disease. "Suppression" refers to administration of the composition after an inductive event, but prior to the clinical appearance of the disease. "Treatment" involves administration of the protective composition after disease symptoms become manifest.

Generally, the selected molecules of the present will be utilised in purified form together with pharmacologically appropriate carriers. Typically, these carriers include aqueous or alcoholic/aqueous solutions, emulsions or suspensions, any including saline

and/or buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride and lactated Ringer's. Suitable physiologically-acceptable adjuvants, if necessary to keep a polypeptide complex in suspension, may be chosen from thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin and alginates.

Intravenous vehicles include fluid and nutrient replenishers and electrolyte replenishers, such as those based on Ringer's dextrose. Preservatives and other additives, such as antimicrobials, antioxidants, chelating agents and inert gases, may also be present (Mack (1982) Remington's Pharmaceutical Sciences, 16th Edition).

The selected molecules of the present invention may be used as separately administered compositions or in conjunction with other agents. These can include various immunotherapeutic drugs, such as cyclosporine, methotrexate, adriamycin or cisplatinum, and immunotoxins. Pharmaceutical compositions can include "cocktails" of various cytotoxic or other agents in conjunction with the molecules of the present invention or even combinations of the molecules of the present invention.

The route of administration of pharmaceutical compositions according to the invention may be any of those commonly known to those of ordinary skill in the art. For therapy, the selected molecules of the invention can be administered to any patient in accordance with standard techniques. The administration can be by any appropriate mode, including parenterally, intravenously, intramuscularly, intraperitoneally, transdermally, via the pulmonary route, or also, appropriately, by direct infusion with a catheter. The dosage and frequency of administration will depend on the age, sex and condition of the patient, concurrent administration of other drugs, counterindications and other parameters to be taken into account by the clinician.

The selected molecules of this invention can be lyophilised for storage and reconstituted in a suitable carrier prior to use. Known lyophilisation and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilisation and reconstitution can lead to varying degrees of functional activity loss and that use levels may have to be adjusted upward to compensate.

The compositions containing the present selected molecules of the present invention or a cocktail thereof can be administered for prophylactic and/or therapeutic

treatments. In certain therapeutic applications, an adequate amount to accomplish at least partial inhibition, suppression, modulation, killing, or some other measurable parameter, of a population of selected cells is defined as a "therapeutically-effective dose". Amounts needed to achieve this dosage will depend upon the severity of the disease and the general state of the patient's own immune system, but generally range from 0.005 to 5.0 mg of selected molecules of the present inventionper kilogram of body weight, with doses of 0.05 to 2.0 mg/kg/dose being more commonly used. For prophylactic applications, compositions containing the present selected polypeptides or cocktails thereof may also be administered in similar or slightly lower dosages.

A composition containing one or more selected molecules according to the present invention may be utilised in prophylactic and therapeutic settings to aid in the alteration, inactivation, killing or removal of a select target cell population in a mammal. In addition, the molecules described herein may be used extracorporeally or in vitro selectively to kill, deplete or otherwise effectively remove a target cell population from a heterogeneous collection of cells. Blood from a mammal may be combined extracorporeally with selected molecules of the invention whereby the undesired cells are killed or otherwise removed from the blood for return to the mammal in accordance with standard techniques.

Assays and Methods Using Agents of the Present Inventions

In yet a further aspect, the present invention provides a method for identifying a molecule involved in mediating syncitial apoptosis, comprising the steps, of: (a) providing a cell population, wherein at least a proportion of the population have one or more syncitia present; (b) examining the one or more syncitia for an altered functional activity of mTOR, and/or p53, and/or the nucleic acid encoding them; and (c) assaying the one or more syncitia, and detecting those one or more molecule/s which interact with mTOR, and/or p53, and/or the nucleic acid encoding them.

Cells

Cells suitable for use according to a method of the present invention include primary cells from sources such as blood. They may also be cell lines such as Hela cells,

Cos cells and so on. Cells are cultured using methods familiar to those skilled in the art and detailed in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d ed. (1989).

In a preferred aspect of the present invention the cells are infected with a virus, more preferably a virus selected from the group consisting of MHV-3, FSV and HIV, even more preferably HIV, most preferably HIV-I.

In some cell types syncitia form spontaneously. Cells within this group include myoblasts which fuse to form muscle fibres, and osteoclasts which forms bone. Alternatively, or in addition, syncitia formation may be induced by viral infection. For example Hela cells transfected with CD4, cultured in the presence of infected H9 cells result in the formation of syncitia after a few days of culture. An example of a protocol is given in Example 1. In a preferred aspect, the cell type selected is capable of syncitia formation.

Modulator Assays

Another aspect of the invention is directed to the identification of agents capable of modulating one or more p53S115P or mTOR molecules. Such agents are herein referred to as "modulators" or "modulating compounds". In a preferred aspect the assay is directed to a compound capable of modulating apoptosis, more preferably syncitial apoptosis.

In an alternative embodiment, the method of the present invention can be used as a negative screening assay. That is, the method can be used to identify those molecules which do not interact with mTOR and/or a binding agent thereof. In this way molecules may be identified which do not cause some or all of the side-effects associated with the administration of mTOR binding agents such as Rapamycin to a patient.

In a preferred aspect, an assay or method of the present invention is used tin connection with a condition involving syncitial apoptosis, a condition selected from the group consisting of: AIDS, cancer, age related bone loss, osteoporosis, leukemia, and skeletal muscle disorders, or a condition involving the cell size related induction of apoptosis.

"Inhibitors," "activators," and "modulators" of p53S115P or mTOR molecules are used interchangeably to refer to inhibitory, activating, or modulating molecules which

can be identified using *in vitro* and *in vivo* assays for p53S115P or mTOR activity and/or expression, *e.g.*, ligands, agonists, antagonists, and their homologs and mimetics.

Modulator screening may be performed by adding a putative modulator test compound to a tissue or cell sample, and monitoring the effect of the test compound on the level of p53S115P or mTOR. A parallel sample which does not receive the test compound is also monitored as a control. The treated and untreated cells are then compared by any suitable phenotypic criteria, including but not limited to microscopic analysis, viability testing, ability to replicate, histological examination, the level of a particular RNA or polypeptide associated with the cells, the level of enzymatic activity expressed by the cells or cell lysates, and the ability of the cells to interact with other cells or compounds. In a particular embodiment, apoptosis can be induced in the treated and untreated cells to determine the effect of the modulator on p53S115P or mTOR apoptosis. Methods for inducing apoptosis are well known in the art and include, without limitation, exposure to chemotherapy or radiotherapy agents and withdrawal of obligate survival factors (*e.g.*, NGF) if applicable. Differences between treated and untreated cells indicates effects attributable to the test compound.

Desirable effects of a test compound include an effect on any phenotype that was conferred by the apoptosis-associated marker nucleic acid sequence. Examples include a test compound that limits the overabundance of mRNA, limits production of the encoded protein, or limits the functional effect of the protein. The effect of the test compound would be apparent when comparing results between treated and untreated cells.

Alternatively, the screening method may include *in vitro* screening of a cell or tissue in which a p53S115P or mTOR molecule is detectable in cultured cells to an agent suspected of inhibiting production of the p53S115P or mTOR molecule; and determining the level of the p53S115P or mTOR molecule in the cells or tissue, wherein a decrease in the level of p53S115P or mTOR molecule after exposure of the cells or tissue to the agent is indicative of inhibition of p53S115P or mTOR molecule production.

The invention also encompasses *in vivo* methods of screening for agents which inhibit expression of the p53S115P or mTOR molecules, comprising exposing a mammal having apoptosis cells in which a p53S115P or mTOR molecule is detectable to an agent suspected of inhibiting production of p53S115P or mTOR molecule; and determining the

level of p53S115P or mTOR molecule in apoptosis cells of the exposed mammal. A decrease in the level of p53S115P or mTOR molecule after exposure of the mammal to the agent is indicative of inhibition of marker nucleic acid expression.

Accordingly, the invention provides a method comprising incubating a cell expressing the p53S115P or mTOR molecule with a test compound and measuring the p53S115P or mTOR molecule level. The invention further provides a method for quantitatively determining the level of expression of the p53S115P or mTOR molecule in a cell population, and a method for determining whether an agent is capable of increasing or decreasing the level of expression of the p53S115P or mTOR molecule in a cell population.

A method for determining whether an agent is capable of increasing or decreasing the level of expression of the p53S115P or mTOR molecule in a cell population comprises the steps of (a) preparing cell extracts from control and agent-treated cell populations, (b) isolating the p53S115P or mTOR molecule from the cell extracts, (c) quantifying (*e.g.*, in parallel) the amount of an immunocomplex formed between the p53S115P or mTOR molecule and an antibody specific to said p53S115P or mTOR molecule.

The p53S115P or mTOR molecules of this invention may also be quantified by assaying for its bioactivity. Agents that induce increased p53S115P or mTOR molecule expression may be identified by their ability to increase the amount of immunocomplex formed in the treated cell as compared with the amount of the immunocomplex formed in the control cell. In a similar manner, agents that decrease expression of the p53S115P or mTOR molecule may be identified by their ability to decrease the amount of the immunocomplex formed in the treated cell extract as compared to the control cell.

mRNA levels can be determined by Northern blot hybridization. mRNA levels can also be determined by methods involving PCR. Other sensitive methods for measuring mRNA, which can be used in high throughput assays, *e.g.*, a method using a DELFIA endpoint detection and quantification method, are described, *e.g.*, in Webb and Hurskainen *Journal of Biomolecular Screening* 1:119 (1996). p53S115P or mTOR molecule levels can be determined by immunoprecipitations or immunohistochemistry

using an antibody that specifically recognizes the protein product encoded by the nucleic acid molecules.

In another aspect of the invention, modulators of p53S115P or mTOR can be identified by monitoring the function of p53S115P or mTOR and secondary genes regulated thereby. As such, p53S115P or mTOR apoptosis can be monitored and correlated to the activity and/or expression of p53S115P or mTOR. Alternatively, the expression and activity of genes regulated by p53S115P or mTOR may be monitored.

Agents that are identified as active in the drug screening assay are candidates to be tested for their capacity to block or promote apoptosis.

The present invention provides a diagnostic assay for predicting predisposition to apoptosis comprising: (i) detecting a level of a molecule selected from the group consisting of an mTOR molecule and a p53 molecule, wherein the p53 molecule is phosphorylated at serine 15; and (ii) comparing the level to a base-line amount.

Any method for analyzing proteins can be used to detect or measure levels of p53S115P or mTOR. As an illustration, size differences can be detected Western blots of protein extracts from the two tissues. Other changes, such as expression levels and subcellular localization, can also be detected immunologically, using antibodies to the corresponding protein. The expression pattern of any cell or tissue types can be compared. Such comparison can also occur in a temporal manner. Another comparison can be made between difference developmental states of a tissue or cell sample.

In another embodiment, p53S115P or mTOR molecules may be selectively detected using an immunological binding assay, *e.g.*, an *in situ* binding assay. In this regard, an antibody which selectively binds to p53S115P or mTOR may be used. More particularly, the antibody may selectively bind to a p53S115P or mTOR.

More particularly, p53S115P or mTOR molecules can be detected and/or quantified using any of a number of well recognized immunological binding assays (*see, e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, *see also* Methods in Cell Biology: Antibodies in Cell Biology, volume 37 (Asai, *ed.* 1993); Basic and Clinical Immunology (Stites & Terr, *eds.*, 7th ed. 1991). Immunological binding assays (or immunoassays) typically use an antibody that specifically binds to a protein or antigen of choice (for example, in this case a p53S115P

or mTOR polypeptide molecule or an antigenic subsequence thereof). The antibody (*e.g.*, anti-p53S115P or mTOR) may be produced by any of a number of means well known to those of skill in the art and as described above.

Immunoassays also often use a labelling agent to specifically bind to, and label the complex formed by the antibody and antigen. The labelling agent may itself be one of the moieties comprising the antibody/antigen complex. Thus, the labelling agent may be a labelled p53S115P or mTOR or a labelled anti-p53S115P or mTOR antibody. Alternatively, the labelling agent may be a third moiety, such a secondary antibody, that specifically binds to the antibody/ΔN p73 complex (a secondary antibody is typically specific to antibodies of the species from which the first antibody is derived). Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (*see, e.g.*, Kronval *et al.*, *J. Immunol.*, 111:1401-1406 (1973); Akerstrom *et al.*, *J. Immunol.*, 135:2589-2542 (1985)). The labelling agent can be modified with a detectable moiety, such as biotin, to which another molecule can specifically bind, such as streptavidin. A variety of detectable moieties are well known to those skilled in the art. A preferred label is a fluorescent label.

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, optionally from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, antigen, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

Generally, immunoassays for detecting a p53S115P or mTOR in a sample may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of antigen is directly measured. In one preferred "sandwich" assay, for example, the anti-p53S115P or mTOR antibodies can be bound directly to a solid substrate on which they are immobilized. These immobilized antibodies then capture the p53S115P or mTOR present in the test sample. The p53S115P or mTOR is thus immobilized, and is then bound by a labelling agent, such as a second p53S115P or

mTOR antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labelled third antibody specific to antibodies of the species from which the second antibody is derived. The second or third antibody is typically modified with a detectable moiety, such as biotin, to which another molecule specifically binds, *e.g.*, streptavidin, to provide a detectable moiety.

In competitive assays, the amount of p53S115P or mTOR present in the sample is measured indirectly by measuring the amount of a known, added (exogenous) p53S115P or mTOR protein displaced (competed away) from an anti-p53S115P or mTOR antibody by the unknown p53S115P or mTOR present in a sample. In one competitive assay, a known amount of p53S115P or mTOR protein is added to a sample and the sample is then contacted with an antibody that specifically binds to the p53S115P or mTOR. The amount of exogenous p53S115P or mTOR protein bound to the antibody is inversely proportional to the concentration of p53S115P or mTOR present in the sample. In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of p53S115P or mTOR bound to the antibody may be determined either by measuring the amount of p53S115P or mTOR present in a p53S115P or mTOR/antibody complex, or alternatively by measuring the amount of remaining uncomplexed protein. The amount of p53S115P or mTOR may be detected by providing a labelled p53S115P or mTOR molecule.

A hapten inhibition assay is another preferred competitive assay. In this assay the known p53S115P or mTOR protein is immobilized on a solid substrate. A known amount of anti-p53S115P or mTOR antibody is added to the sample, and the sample is then contacted with the immobilized p53S115P or mTOR. The amount of anti-p53S115P or mTOR antibody bound to the known immobilized p53S115P or mTOR protein is inversely proportional to the amount of p53S115P or mTOR present in the sample. Again, the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labelled or indirect by the subsequent addition of a labelled moiety that specifically binds to the antibody as described above.

Western blot (immunoblot) analysis may also used to detect and quantify the presence of p53S115P or mTOR in the sample. The technique generally comprises

separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind the p53S115P or mTOR. The anti-p53S115P or mTOR antibodies specifically bind to the p53S115P or mTOR on the solid support. These antibodies may be directly labelled or alternatively may be subsequently detected using labelled antibodies (*e.g.*, labelled sheep anti-mouse antibodies) that specifically bind to the anti-p53S115P or mTOR antibodies.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (*see* Monroe *et al.*, *Amer. Clin. Prod. Rev.*, 5:34-41 (1986)).

One of skill in the art will appreciate that it is often desirable to minimize non-specific binding in immunoassays. Particularly, where the assay involves an antigen or antibody immobilized on a solid substrate it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific binding are well known to those of skill in the art. Typically, this technique involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used with powdered milk being most preferred.

The particular label or detectable group used in the assay is not a critical aspect of the invention, as long as it does not significantly interfere with the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.*, DYNABEADS™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and

colorimetric labels such as colloidal gold or colored glass or plastic beads (*e.g.*, polystyrene, polypropylene, latex, etc.).

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to another molecules (*e.g.*, streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. The ligands and their targets can be used in any suitable combination with antibodies that recognize a p53S115P or mTOR, or secondary antibodies that recognize anti-p53S115P or mTOR.

The molecules can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, *etc.* Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*, luminol. For a review of various labelling or signal producing systems that may be used, *see* U.S. Patent No. 4,391,904.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by

observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labelled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labelled and the presence of the target antibody is detected by simple visual inspection.

The diagnostic and prognostic methods described herein can, for example without limitation, utilize one or more of the detection methods described herein, including but not limited to northern blot analysis, standard PCR, reverse transcription-polymerase chain reaction (RT-PCR), *in situ* hybridization, immunoprecipitation, Western blot hybridization, or immunohistochemistry.

In one aspect, the method comprises *in situ* hybridization with a nucleic acid molecule of the present invention as a probe. This method comprises contacting the labelled hybridization probe with a sample of a given type of tissue potentially containing cancerous or pre-cancerous cells as well as normal cells, and determining whether the probe labels some cells of the given tissue type to a degree significantly different (*e.g.*, by at least a factor of two, or at least a factor of five, or at least a factor of twenty, or at least a factor of fifty) than the degree to which it labels other cells of the same tissue type.

Alternatively, the above diagnostic assays may be carried out using antibodies which selectively detect a polypeptide of the present invention. Accordingly, in one embodiment, the assay includes contacting the proteins of the test cell with an antibody specific for a p53S115P or mTOR and determining the approximate amount of immunocomplex formation. Such a complex can be detected by an assay for example without limitation an immunohistochemical assay, dot-blot assay, and an ELISA assay.

Immunoassays are commonly used to quantitate the levels of proteins in cell samples, and many other immunoassay techniques are known in the art. The invention is not limited to a particular assay procedure, and therefore is intended to include both homogeneous and heterogeneous procedures. Exemplary immunoassays which can be conducted according to the invention include fluorescence polarization immunoassay

(FPIA), fluorescence immunoassay (FIA), enzyme immunoassay (EIA), nephelometric inhibition immunoassay (NIA), enzyme linked immunosorbent assay (ELISA), and radioimmunoassay (RIA). An indicator moiety, or label group, can be attached to the subject antibodies and is selected so as to meet the needs of various uses of the method which are often dictated by the availability of assay equipment and compatible immunoassay procedures. General techniques to be used in performing the various immunoassays noted above are known to those of ordinary skill in the art.

Where tissue samples are employed, immunohistochemical staining may be used to determine the number and type of cells having a p53S115P or mTOR. For such staining, a multiblock of tissue can be taken from the biopsy or other tissue sample and subjected to proteolytic hydrolysis, employing such agents as protease K or pepsin. In certain embodiments, it may be desirable to isolate a nuclear fraction from the sample cells and detect the level of the marker polypeptide in the nuclear fraction.

The tissue samples can be fixed by treatment with a reagent such as formalin, glutaraldehyde, methanol, or the like. The samples are then incubated with an antibody, preferably a monoclonal antibody, with binding specificity for the marker polypeptides. This antibody may be conjugated to a label for subsequent detection of binding. Samples are incubated for a time sufficient for formation of the immuno-complexes. Binding of the antibody is then detected by virtue of a label conjugated to this antibody. Where the antibody is unlabeled, a second labelled antibody may be employed, *e.g.*, which is specific for the isotype of the anti-marker polypeptide antibody. Examples of labels which may be employed include radionuclides, fluorescers, chemiluminescers, enzymes and the like.

Where enzymes are employed, the substrate for the enzyme may be added to the samples to provide a colored or fluorescent product. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art.

In one embodiment, the assay is performed as a dot blot assay. The dot blot assay finds particular application where tissue samples are employed as it allows determination of the average amount of a p53S115P or mTOR associated with a single cell by

correlating the amount of marker polypeptide in a cell-free extract produced from a predetermined number of cells. The diagnostic assays described above can be adapted to be used as prognostic assays, as well.

The methods of the invention can also be used to follow the clinical course of apoptosis. For example, the assay of the invention can be applied to a tissue sample from a patient; following treatment of the patient for apoptosis, another tissue sample is taken and the test repeated. Successful treatment will result in either removal of all cells which demonstrate differential expression characteristic of the apoptosis or pre- apoptosis cells, or a substantial increase in expression of the gene in those cells, perhaps approaching or even surpassing normal levels.

Yet another aspect of the invention provides a method for evaluating the apoptosis potential of an agent by (i) contacting a transgenic animal of the present invention with a test agent, and (ii) comparing the number of transformed cells in a sample from the treated animal with the number of transformed cells in a sample from an untreated transgenic animal or transgenic animal treated with a control agent. The difference in the number of transformed cells in the treated animal, relative to the number of transformed cells in the absence of treatment with a control agent, indicates the apoptosis potential of the test compound.

Another aspect of the invention provides a method of evaluating an anti-apoptosis activity of a test compound. In preferred embodiments, the method includes contacting a transgenic animal of the present invention, or a sample of cells from such animal, with a test agent, and determining the number of transformed cells in a specimen from the transgenic animal or in the sample of cells. A statistically significant decrease in the number of transformed cells, relative to the number of transformed cells in the absence of the test agent, indicates the test compound is a potential anti- apoptosis agent.

The invention will now be described by the following examples which are in no way limiting of the invention.

Example 1

Materials and Methods

Cells lines, transfection, microinjection, and comet assays

HeLa cells transfected with the *Env* gene of HIV-1 LAI (HeLa Env. (27)) and HeLa cells transfected with CD4 (HeLa CD4 (5)) are cultured alone or together (1:1 ratio) in medium supplemented with 10% FCS, essentially as described (in references 5, 6, and 27). Mouse embryonic fibroblasts (MEF) with different genotypes ($p53^{+/+}$, $p53^{-/+}$, $p53^{-/-}$) are obtained from Tyler Jacks. MEF are cultured on coverslips and fused with polyethylene glycol (PEG) (45 sec of incubation with prewarmed [37°C] 50% w/v PEG from Sigma, M.W. 1450, in Ca^{2+}-free PBS, pH 7.2; preceded by two washings with FCS-free medium), followed by 4 washings with complete medium.

N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk; used at 100 μM) is added each 24 hours, whereas other inhibitors were added only once, from the beginning of the culture. Transfection with pcDNA3.1 vector only, pBR322 plasmid constructs containing wild type p53 or mutant (H175, H273) p53 (28) (obtained from T. Soussi), expressed under the control of the cytomegalovirus promoter, luciferase, TOP-luciferase constructs (29) (obtained from J. Chen), or a p53-responsive enhanced green fluorescent (GFP) plasmid (obtained from Klas Wiman) is performed by electroporation (30) 24 hours before co-culture of HeLa CD4 and HeLa Env cells. The p53-responsive GFP plasmid is generated by replacing the luciferase gene in PG13PY Luc (a luciferase construct containing 13 repeats of the p53-binding oligonucleotide 5'CCTGCCCTGGGACTTGCCTGG-3', kind gift from Dr. Bert Vogelstein) with an EcoA7III-MluI fragment from pEGFP-C1.

In certain experiments, all syncytia growing on a pre-marked V-shaped area of a coverslip (>200 per experiment) are microinjected into the cytoplasm (31), with PBS only (pH 7.2), recombinant human Bcl-2 (amino acids 1-218; 500 ng/μl) (32), a mAb specific for mTOR/FRAP (BD Transduction laboratories) or an isotype-(IgG2a) matched LAMP-1 mAb (Oncogene Research Products) (both at 50 ng/μl). Comet assays are performed using a kit from Trevigen (Gaitherburg, MD), on HeLa Env cells (untreatad or treated with 100 μM H_2O_2 for 30 min) or 24-hour-old HeLa Env/CD4 syncytia (non-apoptotic adherent or apoptotic cells).

Patients' samples

Peripheral blood samples are obtained from 49 HIV-infected individuals from the National Institute for Infectious Diseases (IRCCS Lazzaro Spallanzani, Rome, Italy). Patients are selected for this study according to the following criteria: asymptomatic HIV-1 infection (A, CDC 1993), under at least 2 months of antiretroviral therapy interruption, $CD4^+$ T lymphocytes in peripheral blood in the range of 300-600/μl, plasma HIV-RNA levels >80 copies/ml, no treatment with interferons or corticosteroids, absence of infection with hepatitis C virus, hepatitis B virus, and autoimmune disease.

PBMC are isolated by Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) centrifugation of heparinized blood from healthy donors and HIV-seropositive individuals and fixed with 4% formaldehyde in PBS, pH=7.2. Plasma HIV-1 RNA levels are determined by the Nucleic Acid Sequence Based Amplification (NASBA) procedure (HIV-1 RNA QT Assay; Organon Teknika, the Netherlands). Plasma levels of HIV-1 RNA are expressed as HIV-RNA copies/ml with a threshold level of 80 copies/ml. Biopsies of auxillary lymph nodes, obtained from healthy donors and $HIV\text{-}1^+$ asymptomatic patients, *naive* for antiretroviral therapy, are immediately fixed with 10% formalin neutral buffered, dehydrated, and paraffin embedded.

HIV-1 infection

Five x 10^6 HeLa CD4 cells are cultured in the presence of 2.5 x 10^6 chronically HIV-1-infected H9/IIIB cells (obtained from Dr. R.C. Gallo; NIH, Bethesda, MD) in 5 ml of RPMI 1640 supplemented with 10% heat inactivated FCS and 2 mM glutamine. Alternatively, CEM cells containing a plasmid encoding GFP driven by the HIV-1 long terminal repeat ((33); clone 8D6; obtained from J. Corbel, University of California, San Diego, CA) are co-cultured (1:1 ratio) with HIV-1-infected H9/IIIB cells. Cell-to-cell fusion is assessed by determining the GFP-dependent fluorescence (excitation 458 nm, emission 515 nm) in a Fluoroscan plate fluorometer (Labsystems, Helsinki, Finland). Alternatively, cells are resuspended in a solution containing 3.4 mM sodium citrate, 0.05 mg/ml propidium iodide, 0.1 mM EDTA, 1 mM Tris pH 8, and 0.1 % Triton X-100 (34), incubated for 60 minutes at RT, and analyzed for DNA content in a FACScalibur (Becton

Dickinson, San José, CA). This method allows for the quantitation of all apoptotic nuclei, including those of syncytia, which are disrupted by the hypotonic buffer.

$CD4^+$ T cells are purified from freshly isolated PBMC by immunomagnetic negative selection (Stem Cell Technologies, Vancouver, BC, Canada). Cells are cultured for 2 days in RPMI medium supplemented with 10 % fetal calf serum (GIBCO; Madrid, Spain) containing 3 μg/ml phytohemoagglutinin (PHA, Sigma) and 6 U/ml interleukin-2 (IL-2, Boehringer Mannheim). For HIV-1 infection, 10 x 10^6 cells are incubated with the T-cell line-adapted HIV-1 strain IIIB (500 ng of p24) for 4 hours at 37 °C. After washing out unabsorbed virus, cells are cultured at a density of 10^6 cells/ml in RPMI medium containing 20% FCS and 10 UI/ml IL-2 in 24 well plates. Two days after infection, when the first syncytia appear in the infected cultures, infected and uninfected cultures are treated with 5 μg/ml AMD3100, 100 μM ZVAD.fmk, or the indicated dose of rapamycin. Infection is allowed to proceed for two more days. At this time mitochondrial function and nuclear morphology are evaluated after JC1/Hoechst staining as described below. Viral replication is evaluated by measuring the level of HIV-1 core protein p24 in the supernatant (Innogenetics ELISA kit, Barcelona, Spain). Promonocytic U937 cell line are infected at a multiplicity of infection (MOI) of 0.5 $TCID_{50}$ per cell, for 2 h at 37°C washed three times in PBS.

Quantitation of protein expression levels

Protein samples are simultaneously prepared from HeLa Env and HeLa CD4 single cells mixed at a 1:1 ratio in lysis buffer (0 control) or from HeLa Env/CD4 syncytia obtained after 18 or 36 hours of coculture. These samples are then processed by the PowerBlot facility (Becton Dickinson, Lexington, KY), which determines the expression level of approximately 800 different signal-transducing proteins using a combination of SDS-PAGE (5-15% gradient), immunoblotting with specific monoclonal antibodies revealed by a secondary goat anti-mouse horseradish peroxidase, capture of chemiluminesence data by a CDD camera, and computerized processing of densitometric data. Data are normalized by dividing the signal obtained for each protein by the sum of signals obtained for all 800 proteins for one given sample. For proteins revealing a

variation in the expression of at least 20%, determined in two initial runs, expression levels are verified in a third independent experiment.

Immunoblots and co-immunoprecipitation

Aliquots of total protein extracts (40 μg), are run on SDS-PAGE and electroblotted overnight at 4°C onto nitrocellulose membrane. Immunodetection involves antibodies specific for p53, Bax, Bcl-2, p53, mTOR/FRAP (BD Transduction laboratories), p53S15P (Cell Signaling Technology, MA, USA), appropriate secondary antibodies (goat anti-rabbit or goat anti-mouse; Bio-Rad, CA, USA) conjugated to horseradish peroxidase, and the enhanced ECL chemiluminescence detection system (Amersham, UK). Equal loading and transfer is monitored by Ponceau red staining of nitrocellulose membranes. For immunoprecipitation, lysates from sonicated, Triton X-100-solublized cells (60 μg protein in 100 μl PBS with protease inhibitors) are incubated for 90 min at 37°C with 500 ng affinity-purified rabbit polyclonal antibodies specific for p53, p53S15P or Bcl-2, followed by addition of 10 μl packed protein A/G- agarose beads (30 min, 37°C; Santa Cruz Biotechnology), vigorous washing of the pellet (10 min at 10000g, 3 x) in PBS, 5% SDS PAGE, and immunodetection with an mTOR/FRAP-specific mAb.

Fluorescence staining of live cells, immunofluorescence, and immunocytochemistry

For the simultaneous assessment of mitochondrial and nuclear features of apoptosis, live cells are stained with the potentiometric dye 5,5',6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 2 μM, Molecular Probes), as well as Hoechst 33342 (2μM, Sigma) for 30 min at 37°C in complete culture medium essentially as set forth in reference 6. A rabbit antiserum specific for p53S15P is used on paraformaldehyde (4% w:v) and picric acid-fixed (0.19 % v:v) cells and revealed with a goat anti-rabbit IgG conjugated to phycoerythrine (PE) (Southern Biotechnology, Birmingham, AL, USA). Cells are also stained for the detection of Cyt-c (mAb 6H2.B4 from Pharmingen), Hsp60 (mAb H4149 from Sigma), Bax (mAb 6A7, Pharmingen), p21 (mAb EA10, Oncogene Research Products, Boston, MA), MDM2 (mAb IF2, Oncogene Research Products), mTOR/FRAP (mAb 3O, BD Transduction Laboratories), all

revealed by a goat anti-mouse IgG (ALEXA488 conjugate; Molecular Probes), and/or chromatin (Hoechst 33342) (35). Immunocytochemical staining is performed using polyclonal anti p53S15P or monoclonal tTG (Neomarkers, CA, USA) antibodies. A biotinylated goat anti-mouse or anti-rabbit IgG, as a secondary antibody, is used, followed by a preformed horseradish peroxidase-conjugated streptavidin (Biogenex, CA, USA). The reaction is developed using aminoethylcarbazole (AEC) and 3-3' diaminobenzidine (DAB) (Biogenex, CA, USA) as chromogenic substrates and 0.01% H_2O_2. Cells are counterstained in Mayer's acid hemalum. Endogenous peroxidase activity is blocked by preincubation with 3% H_2O_2. Results are quantified by two independent investigators on an average of 500 cells.

Example 2

Early phosphorylation of p53 on serine 15 in HIV-1 Env-induced syncytial apoptosis.

Syncytia formed by coculture of two different HeLa cell lines expressing HIV-1 Env or CD4/CXCR4 spontaneously undergo apoptosis (6, 7). Among a panel of different apoptosis-regulatory proteins, only an upregulation of Bax is detectable 36 hours after syncytium formation (**Fig. 1a**). Significant alterations are also found in the expression level of the pro-apoptotic proteins Bid (which is down-regulated) and caspase-7 (which is upregulated by ~50%). Total p53 levels did not change after formation of syncytia, an increased phosphorylation of serine 15 (but not serines 6, 9, 20, 37, and 397) of p53 (p53S15) is detected, using a panel of phosphoserine epitope-specific antibodies (**Fig. 1b**). Phosphorylated p53S15 (p53S15P) is confined to the nuclei of syncytia (**Fig. 1c**), and is not detected in single cells (SC, **Fig. 1d**). p53S15P could be detected early (6 h) after syncytium formation, well before Bax is overexpressed (Fig. 1a,b) and apoptotic chromatin condensation became detectable (Fig. 1d). Among $p53S15P^+$ syncytia, only a fraction exhibited a conformational change in Bax linked to mitochondrial translocation (**Fig. 1e**) as well as the release of Cyt *c* from mitochondria (**Fig. 1f**). Conversely, the translocation of Bax to mitochondria and of Cyt *c* from mitochondria is only found among $p53S15P^+$ (not $p53S15P^-$) syncytia (**Fig. 1g**). Inhibition of Cyt. *c*-mediated caspase activation by Z-VAD.fmk (**Fig. 1h**) or suppression of Bax translocation by

microinjection of recombinant Bcl-2 protein (36) into syncytia (**Fig. 1i**) fails to affect p53S15 phosphorylation. Both Z-VAD.fmk and Bcl-2 expression did prevent nuclear apoptosis (Fig. 1h,i). p53S15 phosphorylation operates upstream of Bax upregulation/translocation, MMP, and subsequent caspase activation.

Example 3

p53S15 phosphorylation stimulates the expression of p53-transactivated genes in HIV-1 Env-induced syncytia. p53S15 phosphorylation is reported to increase the transactivating function of p53 (37). p53 is not mutated in HeLa cells (39). A low amount of functional p53 in HeLa cells have been reported (41, 42). p53S15 phosphorylation of HeLa syncytia elicited by the Env-CD4 interaction results in transcriptional activation of p53 target genes. Expression of the Bax protein increases (Fig. 1b) and Bax mRNA. Expression levels of p53-activated gene products, MDM2 (**Fig. 2a,c**) and p21 (**Fig. 2b,c**), increase upon syncytium formation. Such enhanced expression is restricted to the p53S15P$^+$ subpopulation of syncytia (**Fig. 2 a,b**). A p53-inducible GFP construct that, when transiently transfected into HeLa Env cells, cultured in the absence of HeLa CD4 cells, fails to be expressed, unless it is co-transfected with p53 (but not with a dominant negative p53 mutant, p53H175) (**Fig. 2d**). Upon coculture with HeLa CD4 cells, this p53-inducible GFP construct is strongly expressed in a subpopulation of p53S15P$^+$ syncytia (but not in p53S15P$^+$ syncytia nor in individual cells) (**Fig. 2e**).

Example 4

p53S15 phosphorylation in HIV-1 infection in vitro and in vivo.

p53S15P is induced by HIV infection *in vitro* and *in vivo*. p53S15P is induced in heterokaryons generated by co-culturing HeLa CD4 cells with a lymphoid cell line chronically infected with a syncytium-inducing HIV-1 isolate (**Fig. 3a**) and in HIV-1-infected U937 myelomonocytary cells (**Fig. 3b**). HIV-1 infection in vitro suffices to induce p53S15P (22). p53S15P is also found among syncytia localized in the T cell area of lymph nodes from HIV-1$^+$ patients (**Fig. 3c**). Such cells also expressed the pre-apoptotic marker tTgase (Fig. 3c). p53S15P is detected in PBMC of HIV-1$^+$ donors but

not in PBMC from HIV-1$^-$ controls (<0.1% positive cells) (**Fig. 3d**). The frequency of p53Ser15P$^+$ PBMC correlates with the percentage of tTgase$^+$ cells (**Fig. 3e**) and viral load (**Fig. 3f**). p53S15P is associated with HIV-1 infection, both upon in vitro infection of human cells as and vivo, in patient-derived samples. p53S15P expression correlates with HIV-1 viremia and pre-apoptosis.

Example 5

p53S15 phosphorylation is mediated by mTOR/FRAP.

p53S15 is phosphorylated by stress kinases, in particular ERK and p38 kinase (43), as well as by several members of the PIKK family, in particular DNA-PK (44), ATM (45) and ATR (46). Among a large panel of serine kinases, phosphatases, and serine kinase activators, the only two proteins whose expression level changed in Env-induced syncytia were mTOR/FRAP, which is upregulated, and the protein phosphatase PP2A, which is down-regulated (**Fig. 4a**). PP2A is reported to be inhibited by mTOR/FRAP (48). PP2A's late (36h) downregulation may be secondary to the upregulation/activation of mTOR/FRAP detectable at 18 h. Rapamycin (a specific inhibitor of mTOR/FRAP) and LY294002 (a general inhibitor of PI-3 kinases including mTOR/FRAP) significantly inhibited p53S15 phosphorylation (**Fig. 4b**). In contrast, the ERK inhibitor PD98059, the p38 inhibitor SB203580, the ATM/DNA-PK inhibitor wortmannin (49, 50) and the ATM/ATR inhibitor caffeine (46, 51) failed to prevent the syncytium-specific p53S15 phosphorylation **(Fig. 4b)**. Accordingly, no phosphorylation of ERK (motif: pTEpY) or p38 kinase (motif: pTGpY) is detectable in syncytia, indicating that these kinases are not activated. Moreover, comet assays did not detect double strand breaks among non-apoptotic p53S15P$^+$ syncytia (**Fig. 4c,d**), further arguing against the implication of DNA-PK, ATM, and ATR (all of which are, in principle, activated by double strand breaks, (44-46)) in p53S15 phosphorylation. As an alternative to rapamycin, microinjection of a monoclonal antibody (mAb) specific for mTOR/FRAP into newly formed syncytia causes a significant inhibition of p53S15 phosphorylation (**Fig. 4e**). p53S15 phosphorylation is mediated via mTOR/FRAP.

Example 6

Cellular alterations induced by activated mTOR/FRAP.

mTOR/FRAP mediates translational control via phosphorylation of the $p70^{s6k}$ protein kinase and the 4E binding protein 1 (4EBP1) (23, 24, 52). In a control experiment performed on single cells, serum withdrawal (which inactivates mTOR/FRAP) is found to cause the dephosphorylation of $p70^{s6k}$ and 4EBP1 (lane 4 in **Fig. 5a**), which are phosphorylated again upon readdition of serum (lane 5). This serum-stimulated phosphorylation is fully inhibited by rapamycin (lane 6) as an internal control of its efficacy. In contrast, both single cells (lane 1) and syncytia (lane 2) cultured in complete, serum-containing medium exhibited constitutive phosphorylation of $p70^{s6k}$ and 4EBP (Fig. 5a), as well as constitutively high expression of a mTOR/FRAP-controlled 5'-terminal oligopyrimidine (TOP)-luciferase reporter construct (**Fig. 5b**). Accordingly, the expression of several mTOR/FRAP-controlled gene products including c-Myc, HIF-1, Rb, Rb2, and STAT3 is not increased upon syncytium formation. Neither the constitutive phosphorylation of $p70^{s6k}$ and 4EBP nor the translation of TOP-luciferase are inhibited by a 24 hour exposure to rapamycin (lane 3 in Fig. 5a and b), and rapamycin did not block the cell cycle of HeLa cells. Within a similar time frame, fusion-dependent p53Ser15 phosphorylation is inhibited by rapamycin **(Fig. 5a)**. mTOR/FRAP co-immunoprecipitated with p53 and p53S15P in syncytia, not in single cells (**Fig. 5c**). Syncytia mTOR/FRAP is enriched in the nucleus, as compared to single cells. This alteration is observed both in $p53S15P^-$ and $p53S15P^+$ syncytia (**Fig. 5d**), and kinetic analyses revealed that the nuclear accumulation of mTOR/FRAP precedes the phosphorylation of p53S15P (**Fig. 5e**). mTOR/FRAP is activated in syncytia, where it phosphorylates p53 within the nucleus.

Example 7

Inhibition of mTOR/FRAP or p53S15 phosphorylation prevents Env-induced syncytial apoptosis.

Rapamycin and LY294002 inhibited signs of syncytial apoptosis in HeLa Env/HeLa CD4 cocultures, including the loss of the $\Delta\Psi_m$ (indicated by a red --> green

shift of the fluorescence emited by the $\Delta\Psi_m$-sensitive fluorochrome JC-1), chromatin condensation (detected with the blue fluorochrome Hoechst 33342) (**Fig. 6a, b**), the translocation of Bax to mitochondria, and the Bax-mediated release of Cyt. *c* from mitochondria (**Fig. 6b**). Rapamycin and LY294002 is associated with the formation of larger syncytia (**Fig. 6c**), an effect quantitatively similar in effect to pan-caspase inhibitor Z-VAD.fmk (**Fig. 6c and (6)**). The apoptosis-inhibitory effect of rapamycin was durable, with a strong inhibitory effect on apoptosis of six-day-old syncytia (81±4% of viable, $\Delta\Psi_m^{high}$ syncytia with 1 μM rapamycin versus only 25±3% $\Delta\Psi_m^{high}$ syncytia in untreated controls, n=3). The anti-apoptotic effects of rapamycin and LY294002 are observed at doses similar to those required for inhibition of p53S15 phosphorylation (**Fig. 4b**). In contrast, wortmannin, caffeine, PD98059, SB203580, CsA, or FK506, which do not affect p53S15 phosphorylation (**Fig. 4b**), also failed to prevent apoptosis (**Fig. 6d**). Thus, a large panel of agents which inhibit apoptosis in other experimental systems failed to prevent syncytial apoptosis, with the notable exception of pyrrolidine dithiocarbamate (PDTC, **Fig. 6g**), a pleiotropic agent known to interfere with p53 function (53, 54). PDTC also reduced the phosphorylation of p53S15 (**Fig. 4b**).

Example 8

p53 participates in syncytial apoptosis.

To assess the role of p53 in syncytial apoptosis, Env and CD4/CXCR4-expressing cells are transiently transfected with two dominant negative (DN) p53 constructs (p53H175 and p53H273) 24 hours prior to fusion. DN-p53 confers protection against all the mitochondrial and nuclear hallmarks of syncytial apoptosis including the translocation of Bax to mitochondria (**Fig. 7**). This protection is partial (~50%), in agreement with the transfection efficiency (~50%). mTOR/FRAP --> p53S15P pathway is associated with the fate of Env-induced syncytia at the pre-mitochondrial level, upstream of the upregulation of Bax. Mouse embryonic fibroblasts (MEF) with different p53 genotypes are subjected to short-term exposure to polyethylene glycol (PEG), resulting in fusion. In wild type MEF ($p53^{+/+}$), syncytium formation leads to phosphorylation of p53S15, positive staining for Bax (**Fig. 8a**), and apoptotic cell death (**Fig. 8b**), whereas syncytia

formed from $p53^{-/-}$ MEF failed to activate Bax (**Fig. 8a**) and to undergo apoptosis (**Fig. 8b**). p53 is rate-limiting for apoptosis triggered by syncytium formation.

Example 9

mTOR/FRAP inhibition prevents syncytial apoptosis induced by HIV-1 infection.

HeLa CD4 cells co-cultured with H9 lymphoid cells chronically infected with the HIV-1 strain IIIb can form syncytia, which undergo apoptosis. Syncytial apoptosis is inhibited by rapamycin, both at the mitochondrial and the nuclear levels (**Fig. 9a, b**). CEM T lymphoma cells engineered to contain a GFP gene under the contorl of the HIV-1 LTR promoter (normally inactive) are cocultured with HIV-1 IIIb-infected H9 cells, in the presence of absenece of rapamycin. Upon formation of syncytia, HIV-1-encoded Tat transactivates LTR and induces GFP expression (33). In the presence of rapamycin (or Z-VAD.fmk, as a positive control) the frequency of viable syncytia is increased, leading to an enhanced GFP-dependent fluorescence (**Fig. 9c**). When individual nuclei from such syncytia are subjected to cytofluorometric DNA content analysis, the frequency of hypoploid (apoptotic) nuclei is found to be strongly reduced by rapamycin (**Fig. 9d**). In a further series of experiments, primary $CD4^+$ lymphoblasts from healthy donors are infected by HIV-1 IIIb *in vitro*, a manipulation that induced syncytium formation and subsequent apoptosis. Addition of rapamycin 48 hours after infection does not affect viral replication, as determined by measuring the production of p24 (**Fig. 9e**). Rapamycin inhibits chromatin condensation and led to an increase in the number of nuclei per syncytium, similar to Z-VAD.fmk (**Fig. 9e**). The cytoprotective effects of rapamycin on lymphoblasts infected with HIV-1 cannot be attributed to an inhibition of viral replication or syncytium formation. Rapamycin inhibits the phosphorylation of p53S15 in HIV-infected cells (**Fig. 9b**). mTOR/FRAP --> p53S15P pathway selectively controls apoptosis of syncytia in human primary $CD4^+$ lymphocytes infected by HIV-1.

Example 10

HIV-1 Env-induced syncytial apoptosis is associated with early changes in gene expression.

Syncytia formed by coculture of two different HeLa cell lines expressing HIV-1 Env or CD4/CXCR4 spontaneously undergo apoptosis (6, 7).

RNA is extracted from samples and analysed by microarray as described below.

HeLa transfected with the Env gene of HIV-1 LAI and HeLa cells transfected with CD4 are resuspended in serum containing culture medium at a concentration of $2x10^6$/ml, and cultured for at 37°C. Samples for RNA extraction are taken at 2, 4, 8 and 16h.

Total RNA isolation

Total RNA is prepared from samples using acid phenol/guanidine isothiocyanate extraction (RNAzol B; Biogenesis), or RNeasy RNA preparation kits (Qiagen). Any contaminating genomic DNA is removed by DNase treatment (DNase I, Gibco-BRL).

Measurement of global gene expression by 'Microarray'

The process of microarraying can be used to profile gene expression of thousands of genes simultaneously. The microarray process is described for the use of Human LifeGrid™microarray filters and can be separated into three parts: the filter, the hybridisation of radiolabelled cDNA probe, and the detection and quantitation of the microarray results.

The microarray filter

This example describes the use of the Human LifeGrid™microarray filters obtained from Incyte Genomics (USA). These filters contain cDNA probes representing approximately 8,400 human mRNAs.

Hybridisation of radiolabelled cDNA probes.

This example describes the synthesis of a radiolabelled cDNA from total cellular mRNA. The labeled cDNA is used to 'probe' DNA fragments, which have been immobilised on to a filter membrane, by complementary hybridisation.

Methodology is as described by manufacturer, for Human LifeGrid™ arrays. Essentially, total cellular RNA (1 μg to 20 μg) or polyA+ mRNA (100 ng to 5 μg) is incubated with an oligo (dT) primer. Primed RNA is reverse transcribed to first strand cDNA in a reaction containing M-MLV reverse transcriptase (RT; alternatively Superscript II is used (Life Sciences)), RT buffer, dNTPs and [α-^{33}P] dCTP (2000-4000 Ci/mmol) at 42°C for 1 to 5hours. Unincorporated nucleotides are removed using spin-columns and the labeled probe stored at -80°C until required.

Labeled probes may also be generated from cDNA, genomic DNA or PCR products. In each case a random primed labeling procedure can be used, for example the Ready-Prime Labeling kit (APBiotech), applied as per manufacturers instructions.

Radiolabelled cDNA probe is hybridised to DNA fragments immobilised onto a membrane (typically a nylon or nitrocellulase filter).

Methodology is as described by manufacturer, for Human LifeGrid™ arrays. Essentially, membrane filters are pre-hybridised in hybridisation buffer (5 to 20 ml) at 42°C for 2 to 16 h using a hybridisation oven (Hybaid). Following pre-hybridisation, the labeled cDNA probe is added to fresh hybridisation buffer (5 to 20 ml) and hybridised at 42°C for 14 to

16 h. Following hybridisation, the hybridisation mix is removed and the filters washed with 2 x SSC buffer at RT for 5 min., twice with 2 x SSC, 1% SDS buffer at 68°C for 30 min. and twice with 0.6 x SSC, 1% SDS buffer at 68°C for 30 min.

Detection and quantitation of the microarray results.

This example describes the use of a STORM Phosphoimager to quantitatively image positive signals across the filter arrays. Hybridised filters are wrapped in plastic wrap (Saran) and exposed to a Low-Energy Phosphoimaging screen (Molecular Dynamics). The screen is then placed on the phosphoimager and the gel image captured by scanning at a resolution of 50 microns.

The captured image file is then analysed using software such as Array Vision (Imaging Research Inc.). In this example we implement analysis with ArrayVision v5.1. This program contains facilities for spot detection and quantification, and background detection and quantification. This data is then exported to a text file for further analysis. A variety of data fields are exported from the ArrayVision analysis, including; Spot Label, Position, Density, Background, and particularly, Background subtracted density (sDens) and signal/noise ratio (S/N). In this example, the exported text file is up-loaded to an SQL-7.0 database, to populate a table containing array data from all experiments. As the data is imported to the database, a Normalisation factor is calculated and the sDENs values modified accordingly. This Normalised data is stored in a newly created column within the table. The Normalisation factor facilitates accurate comparison between datasets. A number of different calculations may be used. A normalization factor may be derived from Linear Regression calculated by reference to housekeeping genes. Alternatively, the Global Mean is calculated as the average of the sDens values across all of the arrays to be compared and a normalisation factor is then derived by division of the overall spot density with the Global Mean value. Spot density values (individual sDens) are then corrected by multiplying across all values with the normalisation factor. In a similar approach a Global Geometric Mean normalization factor may be calculated and

used to adjust the dataset. The data from multiple hybridisation experiments can then be stored in a suitable format, for example in an Access or SQL 7.0 database.

Comparison between arrays generates an output file containing the gene identifier and the fold-change in expression relative to the reference dataset. Fold change, (Tx vs Ty), is calculated by dividing the normalised spot density values of Tx with Ty. In this example, multiple time-course experiments are prepared and fold-change values calculated with reference to the T0 time point.

The fold change data derived from comparison of multiple hybridisation experiments can be analysed using a variety of approaches, including hierarchical clustering, (supervised or unsupervised), k-means clustering or self-organising maps. Software enabling these analyses includes the Cluster and Treeview software (M.Eisen, Stanford Uni, USA), J-Express (European Bioinformatics Institute), GeneMaths (Applied Maths, Belgium) or GeneSpring (Silicon Genetics, USA). In this example hierarchical clustering is implemented using the GeneMaths software. Trees are generated using the WARD algorithm with distance calculated using the Pearson similarity metric. Alternatively Euclidean distance metrics are used.

Simplification of Fold-change data

Following cluster analysis, fold-change data can be difficult to interpret owing to either a very large dataset and/or a wide range in fold change values. The visualization and interpretation of these datasets may be simplified using codes or combined codes. In this example, each unique gene is represented by at least two identical cDNAs on the array. The fold change value is calculated as described, then for each spot, a value above 5-fold change is accorded a code of 2, a fold-change value of less then 5 but greater then 2 is accorded a code of 1 and a fold-change value of less then 2 is accorded a code value of 0. A combined code is then derived by adding the code values for each identical cDNA on the array. The use of combined codes can greatly simplify the Cluster analysis and the subsequent visualisation.

Comparison of coordinate patterns of gene expression, by bioinformatic data analysis, using this model system, allows the identification of cell pathways and processes associated with apoptosis and survival.

In any given experiment or time course, 'differentially regulated' genes (combined code greater than or equal to 2) are identified and clustered by either normalised sDens (level of expression) or by fold chance values. Candidate genes, associated with apoptosis and survival, are those that are reproducibly differentially regulated in multiple experiments or time courses and are additionally 'reciprocally regulated' in conditions that permit apoptosis versus survival, respectively.

Figure 10a shows the visual representation of a clustered selection of candidate apoptosis/survival-associated genes identified on LifeGrid filters. Each row represents the differential regulation of an individual gene. The Fold Change colour scale is shown.

This reveals that Env-induced syncytial apoptosis is associated with significant changes in gene expression (**Fig 10a**).

Among these gene expression changes, transcription elongation factor B (SIII) and forkhead box E1 are significantly upregulated from as early as 4 hours post fusion of syncytia (**Fig. 10b**). Rapamycin has been shown to regulate, not only translation but also transcription levels by suppressing RNA *Pol*I and *Pol*III function (Dennis et al, 1999. Curr Opin Genet Dev 9:49-54). The role of auxillary elongation factors, such as transcription elongation factor B (SIII), is critical to controlling rates of transcription. Forkhead transcription factors are associated with apoptosis and examples control the transcription of pro-apoptotic genes such as Fas. (Brunet A et al, Cell 1999 Mar 19;96(6):857-68).

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What is claimed is:

1. A method for the modulation of apoptosis comprising the step of increasing, decreasing or otherwise altering the functional activity of mTOR/FRAP, or a binding agent thereof.

2. A method for inducing apoptosis comprising the step of increasing, decreasing or otherwise altering the functional activity of mTOR/FRAP, or a binding agent thereof.

3. A method according to claim 1 or claim 2 wherein the apoptosis is syncitial apoptosis.

4. A method according to any of claims 1 to 3 wherein apoptosis is modulated in vivo.

5. The use of mTOR, or an agent which alters the functional activity of mTOR, in the modulation of apoptosis.

6. The use according to claim 5 wherein the apoptosis is syncitial apoptosis.

7. The use according to claim 5 or claim 6 wherein the agent is Rapamycin,

8. A method for identifying a molecule involved in mediating apoptosis, comprising the steps, of:

(a)Providing one or more cells,

(b)Examining the one or more cells for an altered functional activity of mTOR, and/or mTOR and p53, and/or the nucleic acid encoding them,

(c)Assaying the one or more cells, and detecting those one or more molecule/s which interact with mTOR, and/or a binding agent thereof, and/or the nucleic acid encoding them.

9. A method for identifying a molecule involved in mediating syncitial apoptosis, comprising the steps, of:

(a)Providing a cell population, wherein at least a proportion of the population have one or more syncitia present.

(b)Examining the one or more syncitia for an altered functional activity of mTOR, or mTOR and p53 and/or the nucleic acid encoding them,

(c)Assaying the one or more syncitia, and detecting those one or more molecule/s which interact with mTOR, and/or a binding agent thereof, and/or the nucleic acid encoding them.

10. A method according to claim 9 wherein the syncitia are formed by treating the cells with a virus..

11. A method according to claim 10 wherein the virus is HIV-1.

12. A composition comprising mTOR, or mTOR and p53, and a pharmaceutically acceptable carrier, diluent or exipient.

13. The use of mTOR, or binding agent thereof, or mTOR and p53, or a composition according to claim 12, in the treatment of a condition involving syncitial apoptosis.

14. The use according to claim 13 wherein the apoptosis is syncitial apoptosis.

15. The use according to claim 14 wherein the mTOR binding agent is Rapamycin.

16. The use according to claim 12 or claim 13, in the treatment of a condition selected from the group consisting of: AIDS, cancer, age related bone loss, osteoporosis, leukemia, skeletal muscle disorders.

17. The use according to claim 14 wherein the condition is AIDS.

18. The use of mTOR, or a binding agent thereof, or mTOR and p53, in the treatment of a condition involving the cell size related induction of apoptosis.

19. The use where the condition is any one selected from the group consisting of: granulomas, giant cell tumours, giant cell hepatitis and giant cell arthritis.

20. The use of one or more selected from the group consisting of: mTOR, a binding agent thereof, and mTOR and p53 in an assay for identifying an agent which modulates apoptosis and/or the cell size-related induction of apoptosis.

21. A diagnostic assay for predicting predisposition to apoptosis comprising:

(i) detecting a level of a molecule selected from the group consisting of an mTOR molecule and a p53 molecule, wherein said p53 molecule is phosphorylated at serine 15; and

(ii) comparing said level to a base-line amount.

22. A method for identifying a compound capable of modulating p53 serine 15 induction of apoptosis comprising:

(i) obtaining a sample tissue or cell which expresses a p53 molecule;

(ii) exposing said sample to said compound; and

(iii) determining the level of a molecule selected from the group consisting of mTOR and p53, wherein said p53 molecule is phosphorylated at serine 15.

23. A method for identifying a compound capable of modulating mTOR apoptosis activity comprising:

(i) obtaining a sample tissue or cell which expresses a p53 molecule;

(ii) exposing said sample to said compound; and

(iii) determining the level of a molecule selected from the group consisting of mTOR and p53, wherein said p53 molecule is phosphorylated at serine 15.

24. A method for detecting apoptosis in a syncitia comprising detecting an increase in gene expression of transcription elongation factor B (SIII) or forkhead box E1.

25. A method for detecting apoptosis in a syncitia comprising detecting a decrease in gene expression of E1B-55kDa-associated protein 5.

26. A method for modulating apoptosis in a syncitia comprising the step of increasing, decreasing or otherwise altering the functional activity of any one of transcription elongation factor B (SIII), forkhead box E1 or E1B-55kDa-associated protein 5.

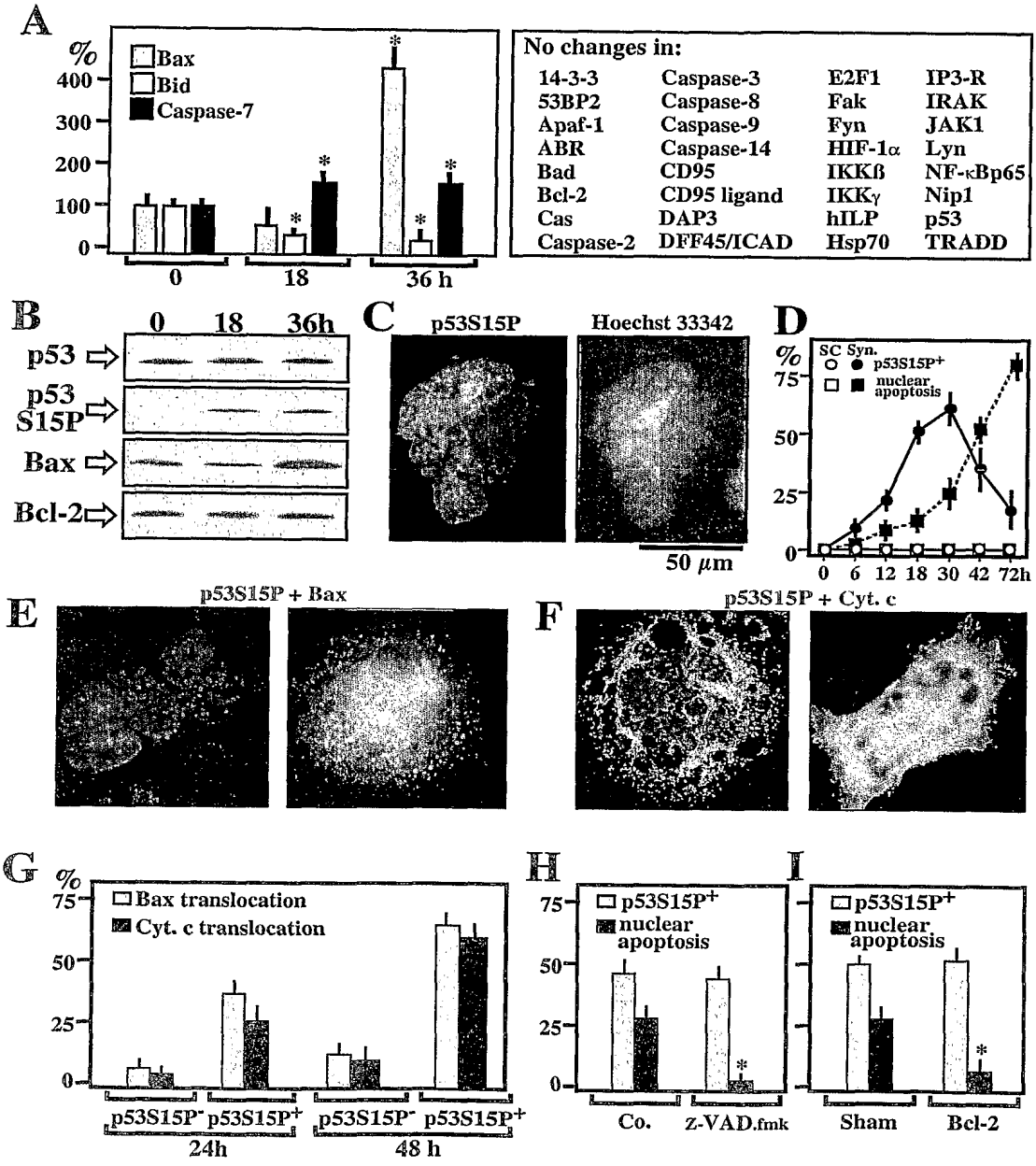
A
%
Bax
Bid
Caspase-7
400
300
200
100
0
0
18
36 h
No changes in:
14-3-3 Caspase-3 E2F1 IP3-R
53BP2 Caspase-8 Fak IRAK
Apaf-1 Caspase-9 Fyn JAK1
ABR Caspase-14 HIF-1α Lyn
Bad CD95 IKKß NF-κBp65
Bcl-2 CD95 ligand IKKγ Nip1
Cas DAP3 hILP p53
Caspase-2 DFF45/ICAD Hsp70 TRADD
B
0 18 36h
p53
p53 S15P
Bax
Bcl-2
C
p53S15P
Hoechst 33342
50 μm
D
SC Syn.
p53S15P+
nuclear apoptosis
%
75
50
25
0
0 6 12 18 30 42 72h
E
p53S15P + Bax
F
p53S15P + Cyt. c
G
%
Bax translocation
Cyt. c translocation
75
50
25
0
p53S15P- p53S15P+ p53S15P- p53S15P+
24h
48 h
H
p53S15P+
nuclear apoptosis
75
50
25
0
Co.
z-VAD.fmk
I
p53S15P+
nuclear apoptosis
Sham
Bcl-2

Fig. 1

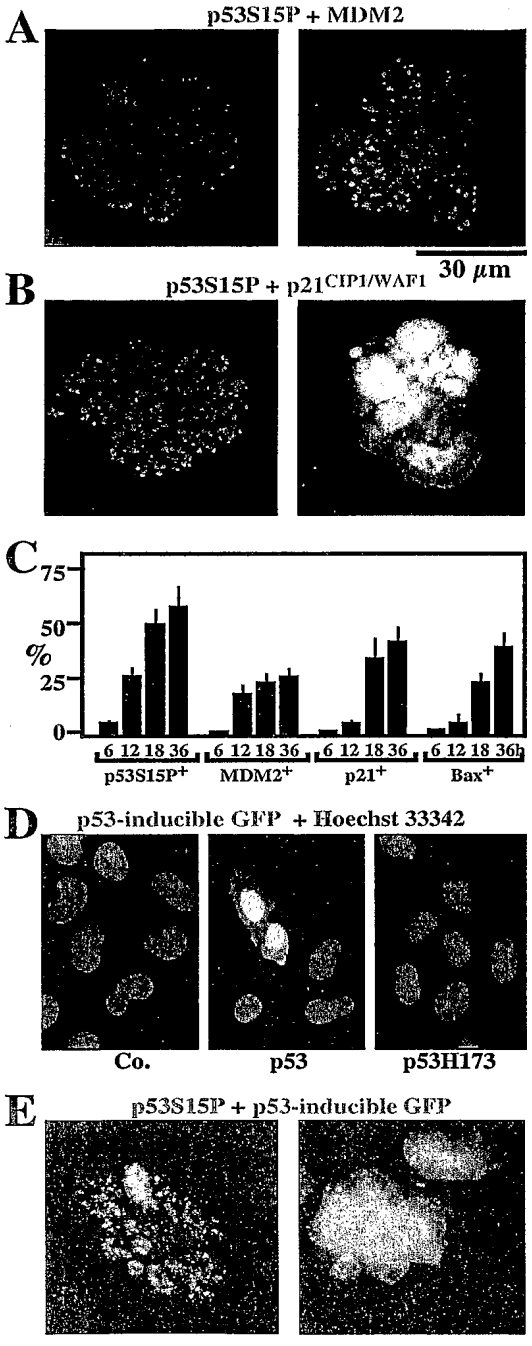
A
p53S15P + MDM2
30 μm
B
p53S15P + p21CIP1/WAF1
C
75
50
%
25
0
6 12 18 36
p53S15P+
MDM2+
p21+
Bax+
D
p53-inducible GFP + Hoechst 33342
Co.
p53
p53H173
E
p53S15P + p53-inducible GFP

Fig. 2

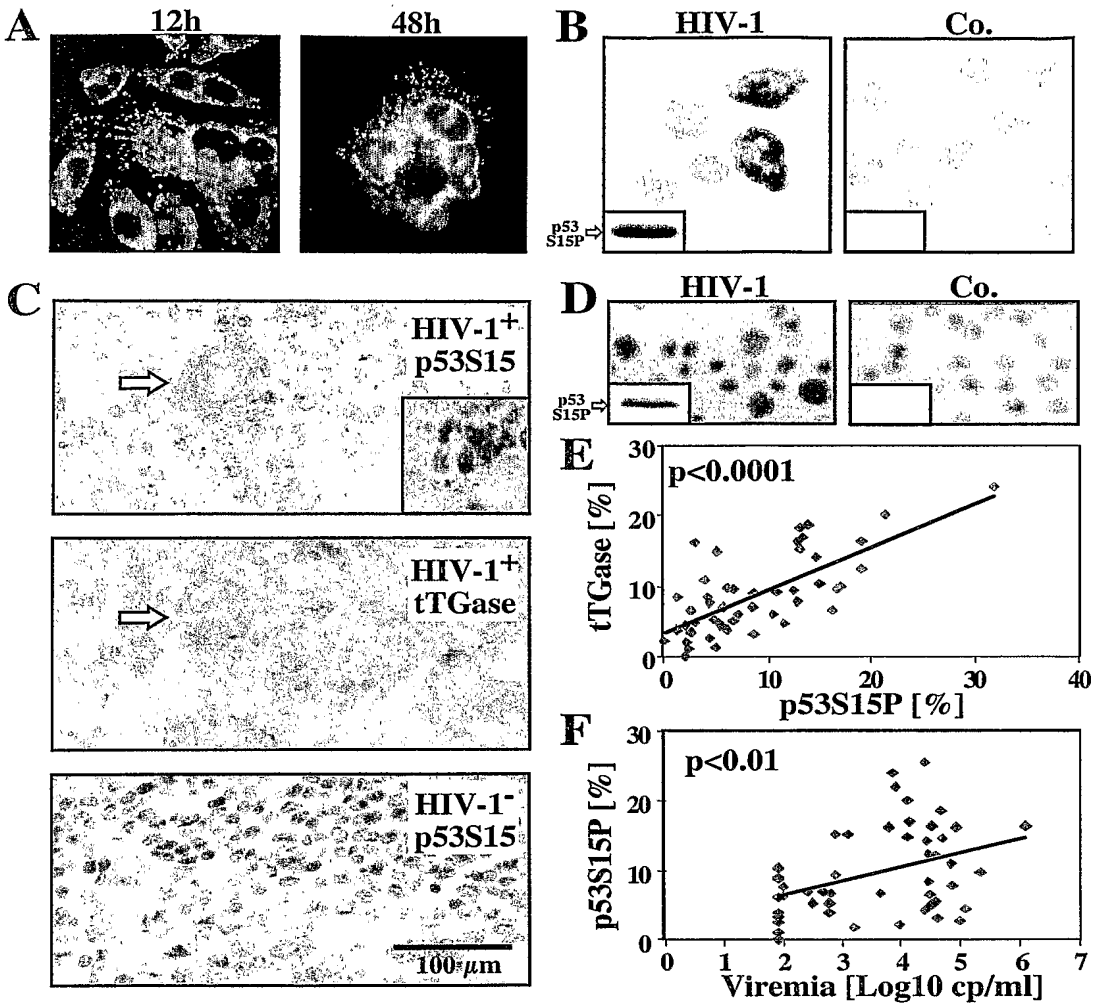
A
12h
48h
B
HIV-1
Co.
p53 S15P
C
HIV-1+ p53S15
HIV-1+ tTGase
HIV-1- p53S15
100 µm
D
HIV-1
Co.
p53 S15P
E
p<0.0001
tTGase [%]
p53S15P [%]
F
p<0.01
p53S15P [%]
Viremia [Log10 cp/ml]

Fig. 3

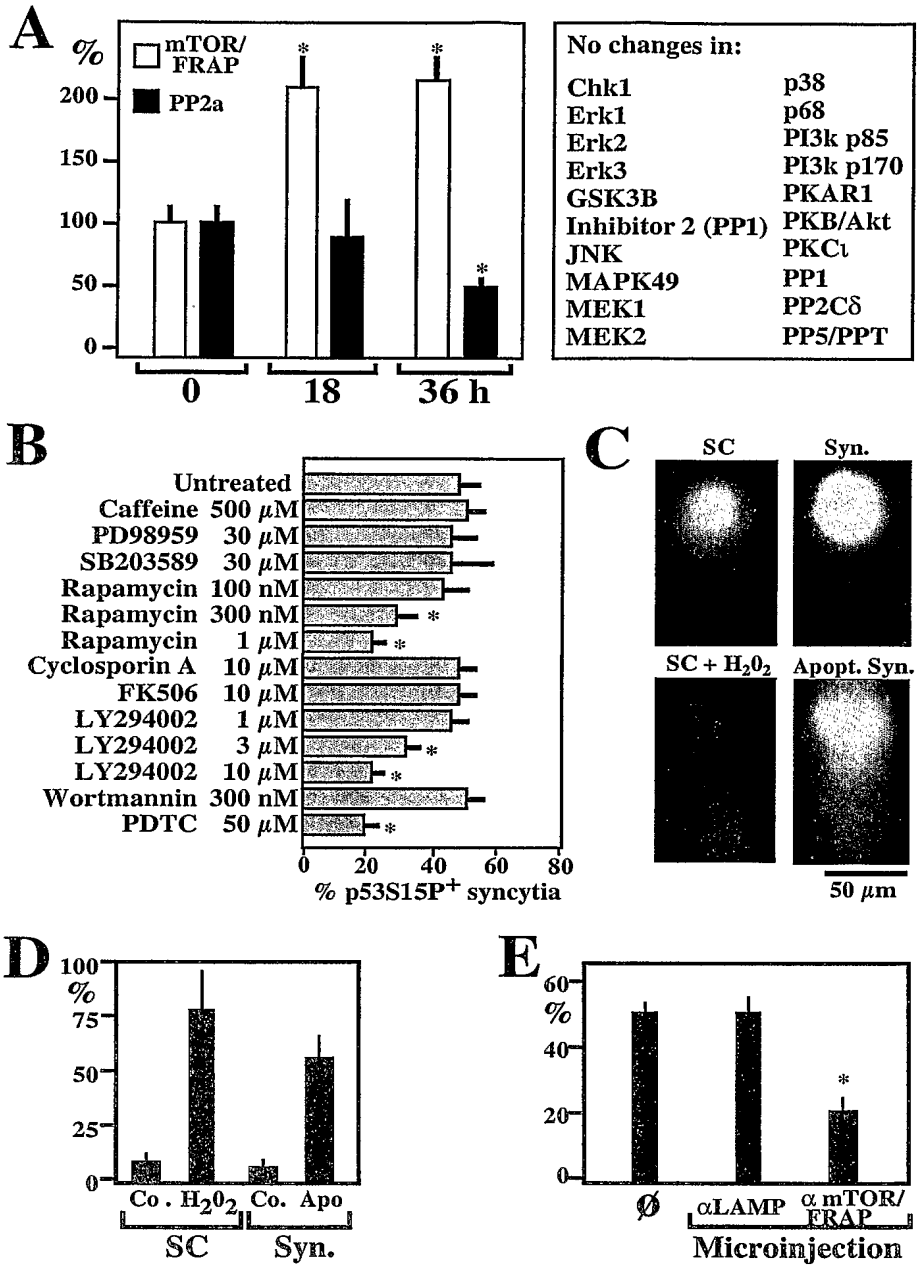
A
%
mTOR/ FRAP
PP2a
200
150
100
50
0
0
18
36 h
No changes in:
Chk1
Erk1
Erk2
Erk3
GSK3B
Inhibitor 2 (PP1)
JNK
MAPK49
MEK1
MEK2
p38
p68
PI3k p85
PI3k p170
PKAR1
PKB/Akt
PKCι
PP1
PP2Cδ
PP5/PPT
B
Untreated
Caffeine 500 μM
PD98959 30 μM
SB203589 30 μM
Rapamycin 100 nM
Rapamycin 300 nM
Rapamycin 1 μM
Cyclosporin A 10 μM
FK506 10 μM
LY294002 1 μM
LY294002 3 μM
LY294002 10 μM
Wortmannin 300 nM
PDTC 50 μM
0 20 40 60 80
% p53S15P+ syncytia
C
SC
Syn.
SC + H2O2
Apopt. Syn.
50 μm
D
100
%
75
50
25
0
Co.
H2O2
Co.
Apo
SC
Syn.
E
60
%
40
20
0
Ø
αLAMP
α mTOR/ FRAP
Microinjection

Fig. 4

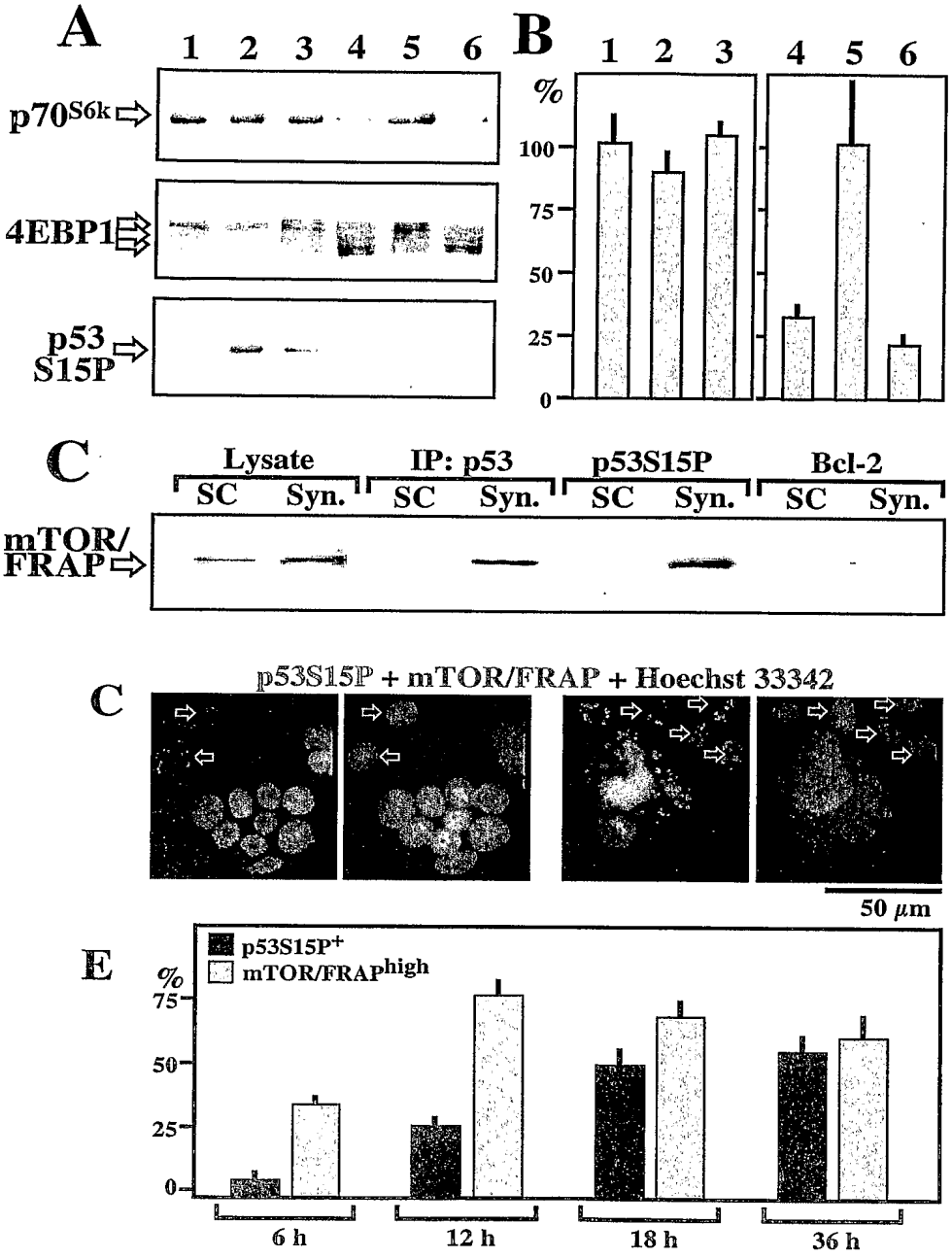
A
1 2 3 4 5 6
p70S6k
4EBP1
p53 S15P
B
1 2 3 4 5 6
%
100
75
50
25
0
C
Lysate
IP: p53
p53S15P
Bcl-2
SC Syn. SC Syn. SC Syn. SC Syn.
mTOR/ FRAP
C
p53S15P + mTOR/FRAP + Hoechst 33342
50 μm
E
p53S15P+
mTOR/FRAPhigh
%
75
50
25
0
6 h
12 h
18 h
36 h

Fig. 5

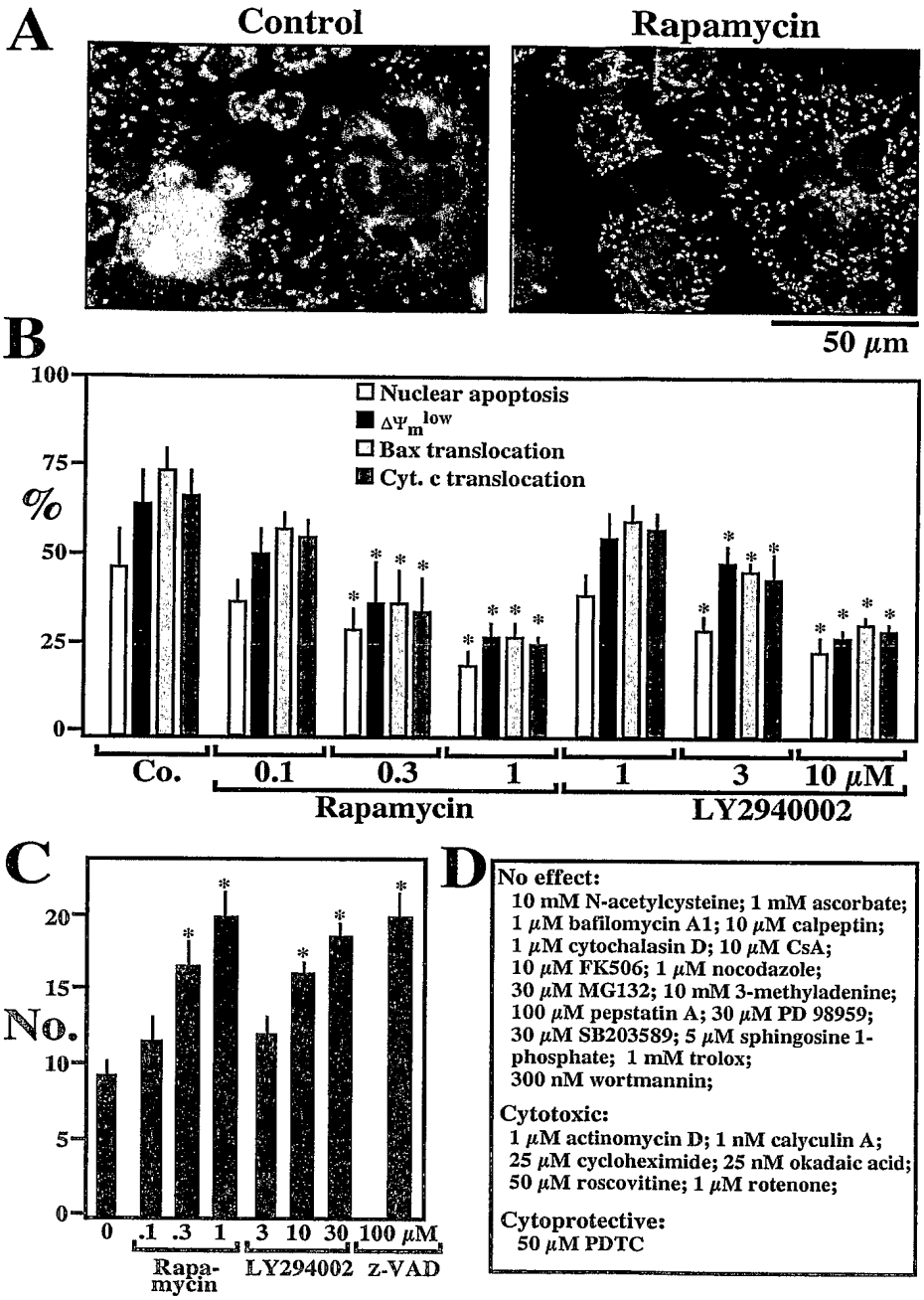
A
Control
Rapamycin
50 μm
B
Nuclear apoptosis
ΔΨm low
Bax translocation
Cyt. c translocation
%
100
75
50
25
0
Co.
0.1
0.3
1
1
3
10 μM
Rapamycin
LY2940002
C
No.
20
15
10
5
0
0 .1 .3 1 3 10 30 100 μM
Rapa-mycin
LY294002
z-VAD
D
No effect:
10 mM N-acetylcysteine; 1 mM ascorbate;
1 μM bafilomycin A1; 10 μM calpeptin;
1 μM cytochalasin D; 10 μM CsA;
10 μM FK506; 1 μM nocodazole;
30 μM MG132; 10 mM 3-methyladenine;
100 μM pepstatin A; 30 μM PD 98959;
30 μM SB203589; 5 μM sphingosine 1-phosphate; 1 mM trolox;
300 nM wortmannin;
Cytotoxic:
1 μM actinomycin D; 1 nM calyculin A;
25 μM cycloheximide; 25 nM okadaic acid;
50 μM roscovitine; 1 μM rotenone;
Cytoprotective:
50 μM PDTC

Fig. 6

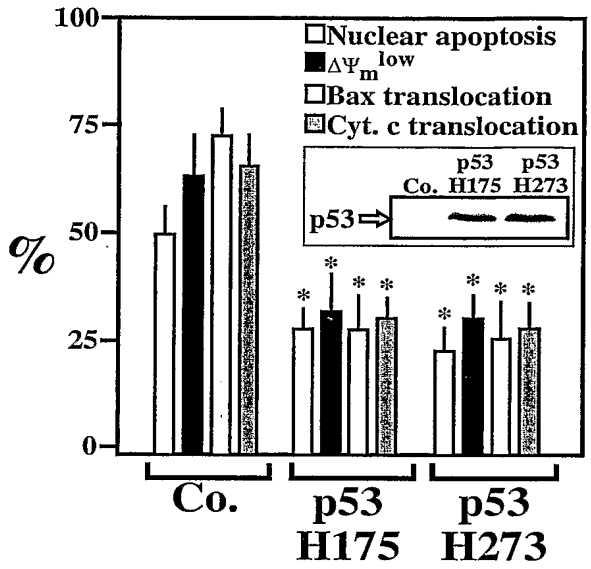
100
75
50
25
0
%
Nuclear apoptosis
ΔΨm low
Bax translocation
Cyt. c translocation
p53
Co.
p53 H175
p53 H273
p53
Co.
p53 H175
p53 H273

Fig. 7

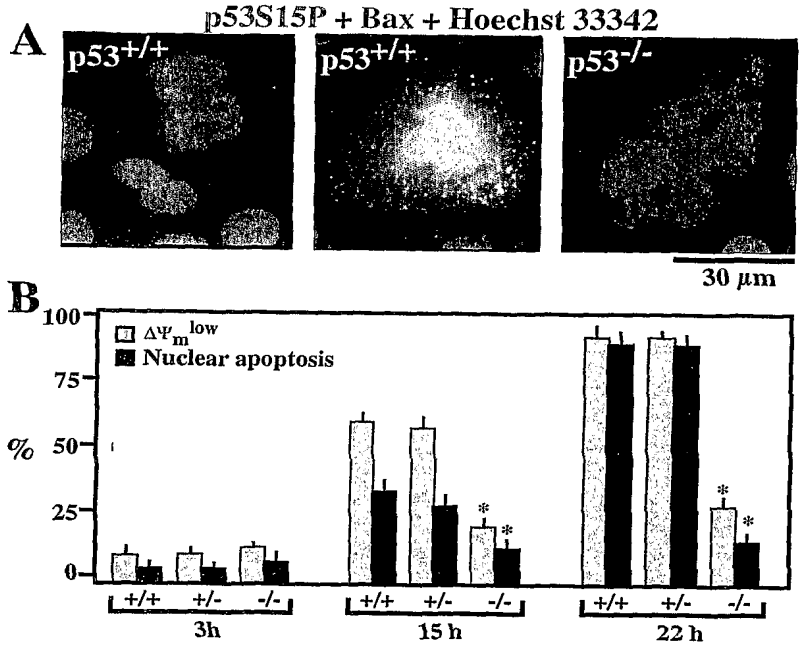
A
p53S15P + Bax + Hoechst 33342
p53+/+
p53+/+
p53-/-
30 μm
B
100
75
%
50
25
0
ΔΨm low
Nuclear apoptosis
+/+ +/- -/-
3h
+/+ +/- -/-
15 h
+/+ +/- -/-
22 h

Fig. 8

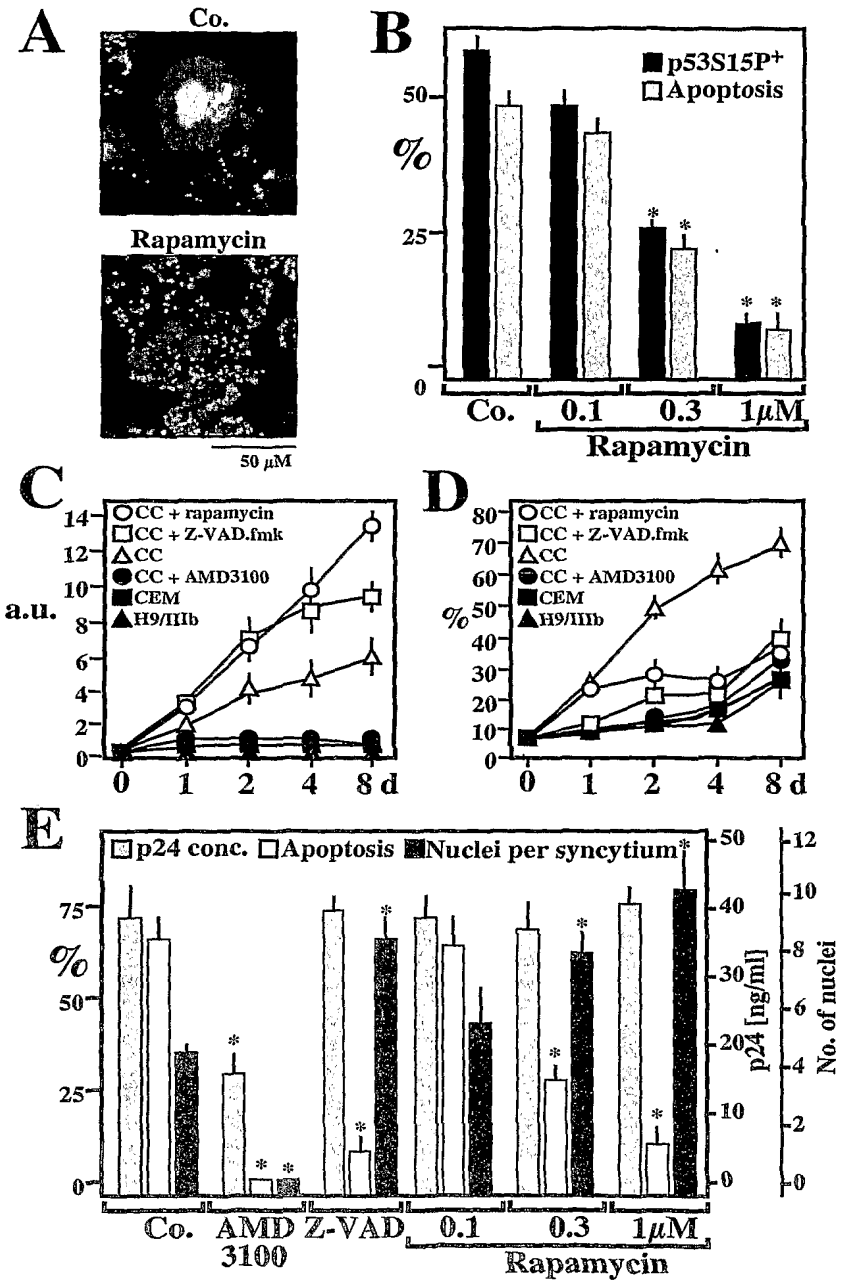
A
Co.
Rapamycin
50 μM
B
p53S15P+
Apoptosis
%
Co.
0.1
0.3
1μM
Rapamycin
C
a.u.
CC + rapamycin
CC + Z-VAD.fmk
CC
CC + AMD3100
CEM
H9/IIIb
0 1 2 4 8 d
D
%
CC + rapamycin
CC + Z-VAD.fmk
CC
CC + AMD3100
CEM
H9/IIIb
0 1 2 4 8 d
E
p24 conc.
Apoptosis
Nuclei per syncytium*
%
p24 [ng/ml]
No. of nuclei
Co.
AMD 3100
Z-VAD
0.1
0.3
1μM
Rapamycin

Fig. 9

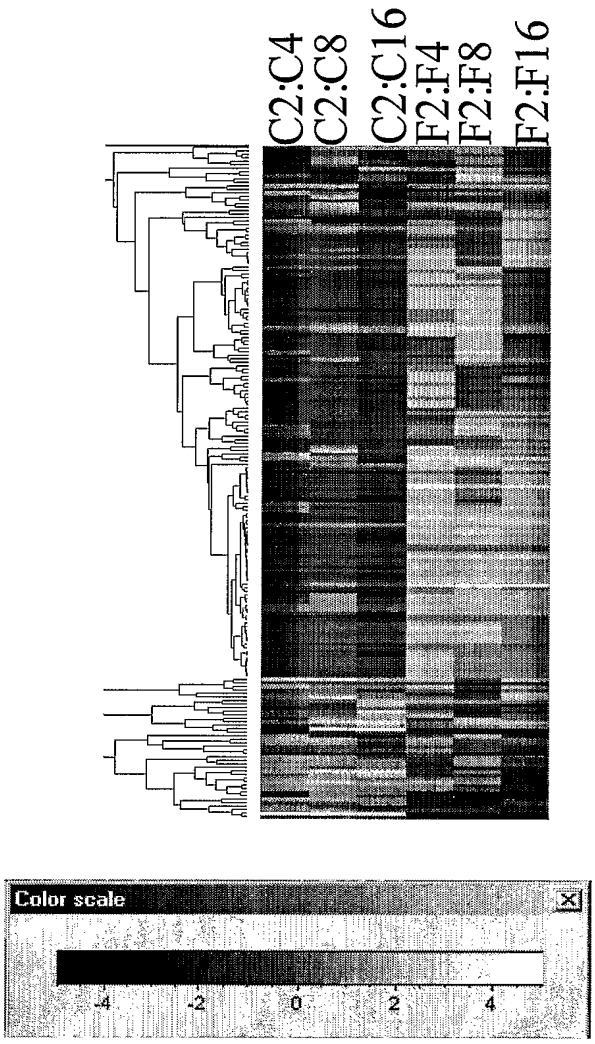

Figure 10

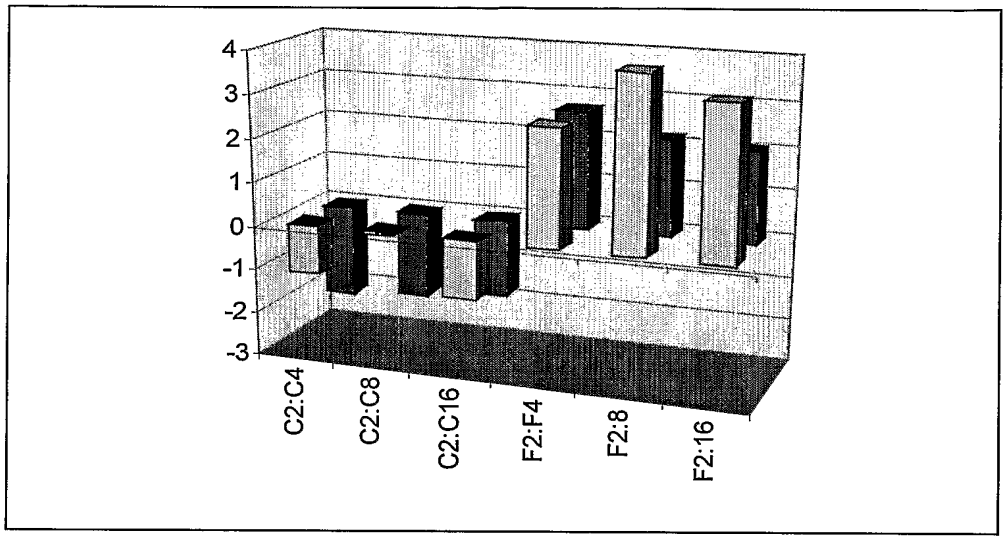

Figure 11

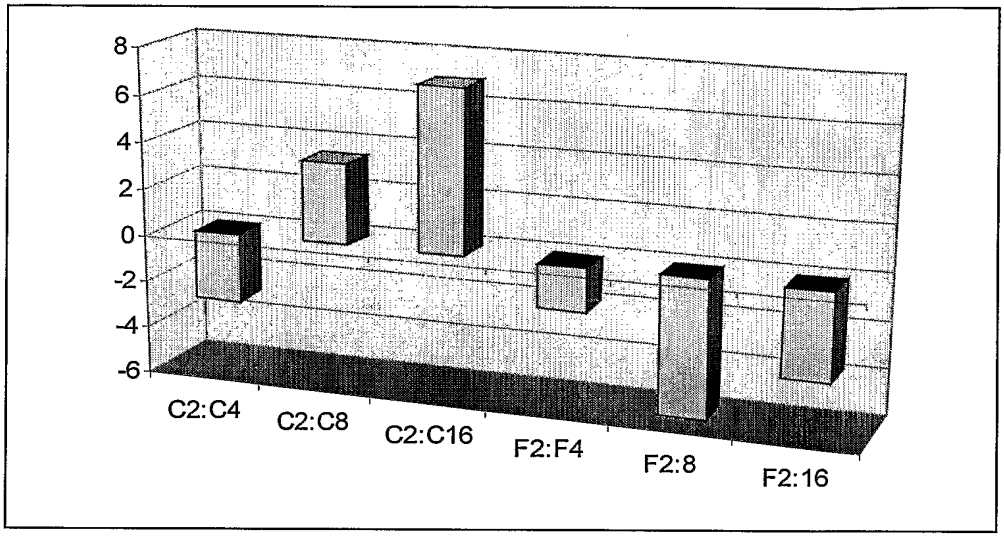

Figure 12